Study on Biocontrol of Phage-Resistant Bacterial Populations

林, 辰諭

Study on Biocontrol of Phage-Resistant Bacterial Populations

LIN CHEN-YU

2024

Study on Biocontrol of Phage-Resistant Bacterial Populations

A thesis submitted by

LIN CHEN-YU

in the Partial Fulfillment of the Requirements for the Degree

of Doctor of Philosophy

at the Department of Bioscience and Biotechnology,

Graduate School of Bioresource and Bioenvironmental

Sciences

Kyushu University, Japan

2024

Table of Contents

Chapter 1. Introduction

1.1. An overview of foodborne pathogens and foodborne illnesses

Food is one of the vehicles for pathogens and plays a vital transmission route of disease due to food-harboring microbial pathogens and environmental contaminations (Gallo et al., 2020). Foodborne diseases are the illnesses engaged with eating contaminated food or beverages, which have a significant impact on food safety and public health globally (Helmy et al., 2023), are among the leading causes of public health issues and result in high mortality rates worldwide (World Health Organization, 2015). Foodborne illnesses occasionally called food poisoning occur as a result of eating food contaminated with pathogenic bacteria, viruses, and toxic or hazardous substances (Ushijima et al., 2014). Food contamination results in 600 million cases of foodborne diseases and 420,000 deaths worldwide every year. 7.69% of the world's population (7.8 billion) experience foodborne diseases every year (Lee and Yoon, 2021). Bacterial foodborne illness accounted for the major food poisoning incidences globally since the pathogens contaminate the foods and then contribute to illnesses in human through the foods. Food is an intricate, dynamic ecosystem, supporting unwanted development, such as food spoilage, a naturally occurring process attributed to undesirable microbial growth and contributing to unacceptable qualities (Iulietto et al., 2015). Despite modern food preservation techniques, there is still 25% of food produced globally wasted from post-harvest to the table due to microbial spoilage (Cenci-Goga et al., 2014).

Microorganisms are crucial for food production, safety and spoilage. While some beneficial microorganisms are essential for food production and giving functions, others could cause foodborne illnesses and spoilage. Microbial interactions with food complexes can lead to food unsuitable for human consumption (Lorenzo et al., 2018). This is because most foodborne microorganisms are naturally pathogenic, causing risks

in food safety and foodborne illnesses to food operators, consumers, and society (Aladhadh, 2023). The major pathogens causing foodborne illnesses are *Salmonella*, *Campylobacter*, and *Escherichia coli* O157:H7 (Food and Drug Administration, 2022). However, foodborne pathogens presented in the environment are normally in low concentrations, blocked by the human immune system, and usually do not cause serious infections. The prevalence of major foodborne illness results from foodborne pathogens directly or indirectly contaminating food using them as a vehicle for oral infection while the food raw material receiving, processing, and transportation (Tauxe, 2002). A foodborne disease outbreak is defined as the occurrence of two or more cases of similar illness resulting from the ingestion of a common food (Bintsis, 2017). Food safety is a critical issue related to the public health and life safety of the citizens and the stability of society (De Jonge et al., 2004). Nonetheless, a growing number of foodborne incidences pose a threat to global regulatory authorities and enhance the requirement for governments, the food industries, and individuals to make more efforts in food safety and prevent foodborne diseases (Bari and Yeasmin, 2018).

Since the detrimental consequences of foodborne pathogen contamination are significant, microbiologists strive to develop novel technologies for the detection and control of foodborne pathogens in food systems. The food system, involving food production, processing, transportation, and consumption, is highly associated with microbial contamination. Numerous methods, including culture-dependent and culture-independent techniques, have been developed for detecting foodborne pathogens in the food supply chain. Strategies based on molecular biotechnology, such as polymerase chain reaction (PCR) and next-generation sequencing (NGS), offer rapid and accurate detection capabilities (Aladhadh, 2023). With the rapid development of genetic technologies, a deeper understanding of large-scale foodborne outbreak cases and the elucidation of foodborne pathogens has been achieved, enhancing our ability to develop more effective food safety policies and practices.

1.2. An overview of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7

Enterohemorrhagic *Escherichia coli* (EHEC) **s**erotype O157:H7 and other Verocytotoxin-producing *E. coli* (VTEC), also known as Shiga-toxin producing *E. coli* (STEC), are zoonotic pathogens associated with outbreaks worldwide (Karmali et al., 2010). Cattle and ruminants carry STEC, contaminating the environment and food sources. Humans get infected through contact or consumption, causing various symptoms (Fig. 1.1). Over 380 different VTEC serotypes have been identified from humans and animals, but only a few serotypes are linked to human disease. EHEC **s**erotype O157:H7, which is accounted for the major *E. coli* food poisoning outbreaks in the United States (US) (Sperandio and Nguyen, 2012), is a bacterial pathogen that causes bloody diarrhea, contributing to a high risk of hemolytic-uremic syndrome (HUS) (Wang et al., 2017). In the US, about 96,000 illnesses are attributed to EHEC O157:H7 annually (Scallan et al., 2011). In Japan, EHEC infection leads to approximately 4,000 foodborne illnesses cases, comprising 2,500 symptomatic and 1,500 asymptomatic cases annually (Furukawa et al., 2018).

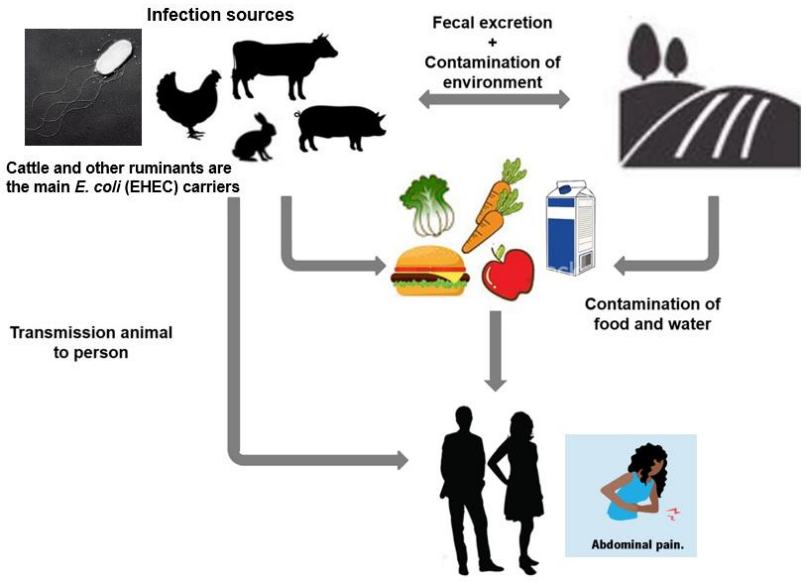


Fig. 1.1. Diagram illustrating the various sources of contamination and pathways of Shiga toxin-producing *Escherichia coli* infection transmission influenced by diverse environmental factors.

EHEC serotype O157:H7, has the basic characteristics of *E. coli* which is a facultative anaerobic gram-negative rod bacterium, without spore, flagellated that is widespread in the environment but is also an enteric bacterium and inhabits the large intestine of mammals. Generally, *E. coli* is endemic and non-pathogenic in humans. However, *E. coli* that cause enteritis are known as pathogenic *E. coli*, which are grouped based on their mechanism of causing symptoms. There are six types of pathogenic *E. coli*: enteropathogenic, enterotoxigenic, verocytotoxigenic (which included enterohaemorrhagic), enteroinvasive, enteroaggregative and diffusely adherent, all of which are orally transmitted through food and drinking water (Percival and Williams, 2014). Among pathogenic *E. coli*, the most common and most dangerous organism is EHEC serotype O157:H7. Additionally, EHEC is known for its strong resistance and ability to survive in the external environment. It can tolerate low temperatures and survive in the refrigerator for a long period, up to 9 months in ground beef at -20 °C and shows no significant change in viability at -80 °C. EHEC can grow rapidly at temperatures ranging from 30 °C to 42 °C. However, the bacterium shows poor growth from 44 °C to 45 °C and does not grow over 45 °C. EHEC adapts oral-fecal lifestyle in cattle and other ruminants. EHEC has an intricate acid resistance (AR) system that enables it to survive through the acidic environment of the stomach (Tuttle et al., 1999).

In 1982, EHEC serotype O157:H7 was firstly recognized as a human pathogen, and then several studies reported that the prevalence of EHEC O157:H7 infections has increased dramatically since the 1990s (Clarke, 2001). The tendency of gradual increase of EHEC O157:H7 infections has become a global public health problem due to their strong pathogenicity, lethality, and the risk of using antibiotic treatment exacerbating the Shiga toxin-mediated cytotoxicity (Nguyen and Sperandio, 2012).

1.2.1. Methods for control of EHEC O157:H7

Globally, the primary pathways through which EHEC O157:H7 is transmitted include poultry, livestock, and products derived from meat. Additionally, this bacterium

has the capability to persist outside its host, which can result in the pollution of fresh water sources as well as fruits and vegetables. Historically, the majority of outbreak incidents have been linked to the ingestion of beef that was not fully cooked or milk that was consumed raw (Doyle, 1991). Despite the notable resistance of EHEC to a range of stressful conditions, studies have shown that the pathogen can be effectively inactivated in ground beef when heated at 60°C for a duration of 45 seconds (Ugorakova and Bukovska, 2003). Moreover, its ability to endure in various environments can be significantly reduced by a combination of factors, such as temperature fluctuations, dehydration, the presence of native microbial populations, variations in moisture levels, and exposure to oxygen-rich atmospheres. In response to the challenges posed by EHEC O157:H7 in food products, the food industry has employed various control strategies, including physical methods like irradiation, pasteurization, pulsed electric field, and high-pressure processing, as well as chemical tactics involving sanitizers such as peroxyacetic acid, chlorine dioxide, sodium hypochlorite, and organic acids like acetic, lactic, and citric acid. While these approaches are effective in reducing EHEC O157:H7 levels in foods, they often come with environmental drawbacks and can adversely affect the food's intrinsic qualities, such as sensory properties, and pose toxicity risks. Furthermore, the increasing consumer demand for chemical-free and organic foods has led to a decrease in the acceptance of chemical additives in food products. Consequently, there's been a significant shift towards exploring alternative methods that are both safe for human consumption and environmentally friendly. Biocontrol agents like bacteriophages, probiotics, plant-derived natural compounds, bacteriocins, endolysins, and enzymes are emerging as promising solutions, offering a way to manage food safety concerns without compromising on environmental sustainability or food quality (Puligundla and Lim, 2022). On the other hand, the reduction of EHEC O157:H7 prevalence in cattle through interventions such as vaccination holds significant potential for decreasing the

incidence of foodborne diseases in humans. Vaccination strategies targeting cattle can inhibit intestinal colonization and reduce fecal excretion of EHEC O157:H7, which is a critical step in preventing the transmission of this pathogen from cattle to humans via contaminated food products. Studies exploring the effectiveness of these interventions in cattle and their impact on reducing human foodborne illnesses highlight the importance of controlling EHEC O157:H7 at the pre-harvest level (Saeedi et al., 2017).

In clinical therapy, while studies have shown EHEC O157:H7 is sensitive to various antibiotics like nalidixic acid and ampicillin (Dontorou et al., 2003), using these medications for treating infections doesn't improve the course of the illness. In fact, it could worsen conditions by increasing the risk of hemolytic uremic syndrome (HUS), attributed to the enhanced release of Shiga toxins by the bacteria. This underscores the complexity of treating EHEC infections and the need for cautious therapeutic approaches. Moreover, antibiotic resistance in EHEC O157:H7 often results from mutations, contributing to the broader issue of antibiotic failure, a significant global health threat. Antimicrobial resistance is a severe global issue, causing nearly 5 million deaths annually and is on the path to become the primary cause of death (Fig. 1.2).

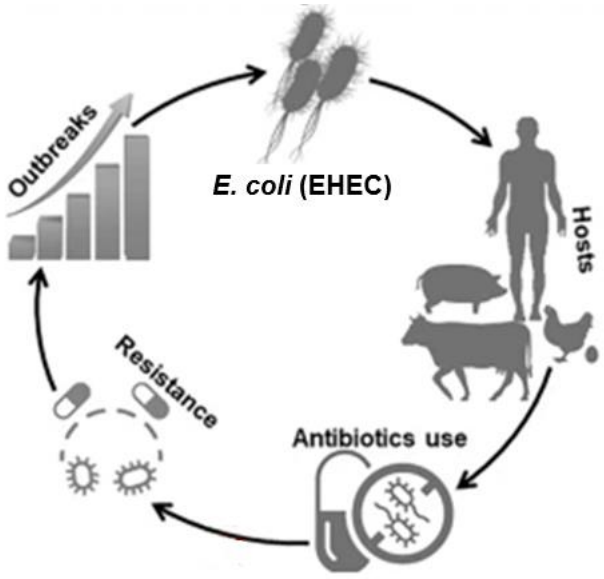


Fig. 1.2. The diagram shows the emergence of multiple-drug-resistant EHEC caused by the abuse of antibiotics.

This crisis is exacerbated by natural evolution and widespread use of antibiotics. The antibiotics, when released into environments such as untreated wastewater, increase selective pressure for resistant strains. The escalating challenge of antimicrobial resistance signals a looming era where existing antibiotics may fail to combat infections effectively, presenting a critical global health concern. (Gambushe et al., 2022). As a response, research is increasingly focused on bacteriophages (Viruses that attack bacteria) as a potential strategy to combat bacterial pathogens, offering a targeted approach to addressing antibiotic-resistant infections.

1.2.2. Phages as potential biocontrol agents of EHEC O157:H7

Bacteriophages (Phages) are viruses that are widely present in the environment. Because phages only target and lyse their hosts, making them effective for controlling bacterial infections even before antibiotics were developed. (Azam et al., 2021). Recently, their ability to selectively eliminate foodborne pathogens without harming beneficial bacteria has renewed interest in their application (Kazi and Annapure, 2016). Owing to their high host specificity, phages can selectively kill host bacteria and therefore do not affect other beneficial bacteria present in food. Furthermore, phages are considered harmless to humans as they specifically attack their host bacteria. Utilizing lytic phages offers a promising approach to combat drug-resistant bacteria. This method of microbial control not only proves effective but also stands out as a cost-efficient alternative to antibodies, with the potential for large-scale production (Bárdy et al., 2016; Labrie et al., 2010). Bacteriophage biocontrol, leveraging phages' natural lytic cycle to eliminate harmful bacteria, is gaining recognition as an eco-friendly approach (Moye et al., 2018) (Fig. 1.3). It aligns with consumer preferences for chemical-free foods, allowing for "Clean-Label" product claims. The U.S. FDA's approval of ListShield™ marked the first phage-based product recognized as a GRAS (Generally recognized as safe) food additive, leading to an increase in regulatory approvals globally for phage applications in food safety, including against EHEC

O157:H7 (Table 1). Following the U.S., other nations including Israel, Canada, Switzerland, Australia, New Zealand, and the EU have approved phage products for food use, reflecting a growing international consensus on the potential of phages to enhance food safety. (Moye et al., 2018). However, the concern about the emergence of phage-resistant bacterial isolates is a significant technical challenge in the application of phage biocontrol strategies for enhancing food safety (Hong et al., 2016). An example of this is seen with PhageGuard Listex™ P100, aimed at combating *Listeria monocytogenes*—a highly concerning pathogen in the food sector due to its severe health implications for humans. The emergence of resistant *Listeria* strains in facilities within Austria has been linked to the adaptation of the bacteria to the phages found in this product. This adaptation is a form of survival strategy, where bacteria modify their surface receptors or implement different methods to avoid being infected by phages, a process that is part of natural evolution (Fister et al., 2016).

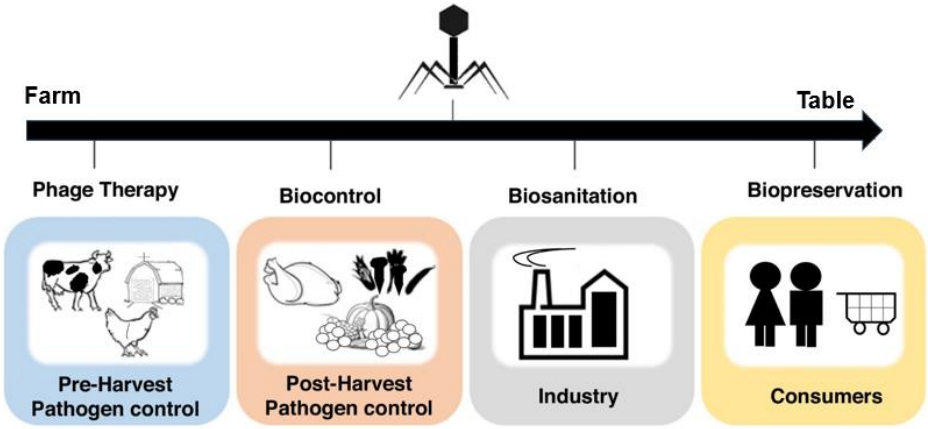


Fig. 1.3. Applications of bacteriophages in food safety at each stage of the farm-to-table process (Endersen and Coffey, 2020).

For therapeutic phage applications in clinical, with its history dating back to the early 20th century, presents a viable alternative to conventional antibiotics due to the unique advantages it offers, particularly its specificity to target pathogens while sparing the beneficial microbiota. Three critical requirements are established: Phages must only engage in the lytic cycle to prevent unwanted genetic integration into host bacteria,

which helps avoid the spread of harmful genes like those conferring antibiotic resistance. They need to exhibit specific antimicrobial effectiveness against designated pathogens, necessitating their precise selection or engineering. Additionally, it's essential to purify phage formulations from bacterial by-products and toxins to minimize adverse effects on patients, ensuring safety by keeping endotoxin levels within acceptable limits (Sarhan and Azzazy, 2015). A successful example, SNIPR Biome's SNIPR001, a CRISPR-armed phage therapeutic, has shown promising interim clinical results in a phase I trial. It's designed to specifically target and eliminate *E. coli*, including antibiotic-resistant strains, from the human gastrointestinal tract. SNIPR001 effectively reduced gut *E. coli* levels and is now being considered for further studies to evaluate its impact on reducing infections in cancer patients at high risk of *E. coli* bloodstream infections. This marks a significant advancement in using phage therapy and CRISPR technology to combat antibiotic resistance (Gencay et al., 2024). However, similar to the issue observed with antibiotics, the evolution of bacterial resistance to phages could limit the long-term sustainability of phage-based treatments. Phage therapy also faces the inevitable threat of phage resistance, which can limit its efficacy. Resistance development occurs through diverse mutations that culminate in impaired adsorption of phages to their bacterial targets. Strategic selection of phage combinations that impose high genetic barriers to resistance is suggested as a mitigation strategy. The in vitro characterization of phage-resistant mutants may help optimize therapeutic phage selection and cocktail design for improved safety and efficacy (Hesse et al., 2020). Understanding phage-host interactions, including resistance mechanisms and their consequences on bacterial fitness, is essential. Detailed information on these interactions can aid in overcoming challenges posed by phage resistance during therapy. Phage resistance often results from mutations in genes involved in the synthesis or assembly of cell surface structures, highlighting the complexity of phage-host dynamics (Li et al., 2022).

Table 1. The phage-derived products approved by the regulatory authorities for food safety applications (Moye et al., 2018).

Product Name	Target pathogen	Regulatory
Ecolicide® (EcolicidePX™)	*E. coli* O157:H7	USDA, FSIS Directive 7120.1
EcoShield™	*E. coli* O157:H7	FDA, FCN 1018; Israel Ministry of Health; Health Canada
ListShield™	*L. monocytogenes*	FDA, 21 CFR 172.785; FDA, GRN 528; EPA Reg. No. 74234-1; Israel Ministry of Health; Health Canada
SalmoFresh™	*Salmonella* spp.	FDA, GRN 435; USDA, FSIS Directive 7120.1; Israel Ministry of Health; Health Canada
ShigaShield™ (ShigActive™)	*Shigella* spp.	FDA, GRN 672
PhageGuard Listex™	*L. monocytogenes*	FDA, GRN 198/218; FSANZ; EFSA; Swiss BAG; Israel Ministry of Health; Health Canada
	E. coli O157:H7	FDA, GRN 757 pending as of 19 March 201
Finalyse®	*E. coli* O157:H7	USDA, FSIS Directive 7120.1
AgriPhage™	*Xanthomonas campestris* pv. vesicatoria, *Pseudomonas syringae* pv. tomato	EPA Reg. No. 67986-1
SalmoPro®	*Salmonella* spp.	FDA, GRN 603
	Salmonella spp.	FDA, GRN 752 pending as of March 19, 2018

1.3. An overview of phage resistance

The use of phages with these advantages could be promising for the control of food poisoning bacteria in improving food quality as well as in clinical therapy. However, faced with naturally existing phage predators, bacteria have evolved various mechanisms to prevent phage infection and reproduction. Prolonged contact between phages and bacteria has been reported to lead to the emergence of viable bacteria that are resistant to phages (Labrie et al., 2010). We are currently in a golden era of discovering defense mechanisms against phages. Beyond the intrinsic fascination with these defense systems and the transformative applications that have emerged from them, such as CRISPR-Cas9 and restriction endonucleases, the role of such defense systems in developing resistance to phages, particularly in clinical environments, remains largely unexplored. Bacteria have developed sophisticated methods to acquire resistance, either through genetic mutations that enhance survival or by obtaining DNA from an already resistant bacterium. Among the mechanisms that afford bacteria adaptive immunity, CRISPR-Cas systems (Clustered regularly interspaced short palindromic repeats–CRISPR associated proteins) stand out. These systems are instrumental in the evolutionary journey of prokaryotes, with horizontal gene transfer (HGT)—via transformation, conjugation, and transduction—playing a pivotal role. HGT can influence bacterial fitness in various ways, being either beneficial, neutral, or harmful (Goyal, 2022).

The CRISPR-Cas adaptive immune systems are particularly noteworthy for their role in managing gene transfer through conjugation and transformation, serving as a line of defense against unwanted genetic material. However, bacterial defenses against phages, including those mediated by CRISPR, come at a significant cost to the growth rate. These defenses can be quickly abandoned when the threat from pathogens diminishes. The dilemma of how bacteria maintain their molecular defenses, despite their high costs, in environments where pathogen levels fluctuate and competition

among strains is fierce, poses a significant challenge to our understanding of evolutionary biology. In their natural habitats, bacteria face relentless selective pressures, propelling both bacteria and phages into an evolutionary arms race. This contest is marked by rapid mutation rates and the facilitation of horizontal gene transfer, accelerating the evolution of genetic characteristics and promoting genetic diversity. This dynamic occurs because when one entity develops a strategy to bypass the other's defenses, it prompts the opponent to adapt and circumvent this new defense mechanism. As a result, bacteria have developed numerous defense strategies to shield themselves against phages. Conversely, phages have evolved sophisticated countermeasures to bypass these bacterial defenses. When phages infect bacterial cells, they encounter a variety of antiviral mechanisms within bacterial populations. The resistance strategies that bacteria employ to dodge phage infections can be broadly categorized into three groups: (1) Receptor Adaptations: This category includes random mutations or phenotypical changes in bacteria that lead to reduced phage adsorption. Such alterations make it more difficult for phages to attach to and invade bacterial cells, as these adaptations affect the receptors phages target for entry. (2) Host Defense Systems: These are specialized molecular pathways that bacteria have evolved specifically to fend off or mitigate phage infections. This category encompasses a range of mechanisms, from restriction-modification systems to the CRISPR-Cas system, which provide bacteria with the means to detect and destroy phage genetic material. (3) Phage-derived Phage Defense Systems: These are unique molecular pathways encoded by phages themselves that help the host bacteria compete against other invading phages. This interesting twist in the evolutionary arms race benefits the host by providing it with additional defense tools against competing phages, thereby ensuring the survival and propagation of the phage that encodes these beneficial systems (Egido et al., 2022).

Despite the wide variety of phage resistance mechanisms displayed by bacteria, phages have countered with an equally diverse array of strategies. This evolutionary

back-and-forth means that for all defense mechanism bacteria develop, phages evolve countermeasures to overcome these barriers. This dynamic interaction underlines the intricate and ongoing co-evolutionary battle between phages and bacteria, with each side continually adapting to the other's moves. The intricate balance of attack and defense in this microscopic arms race highlights the complexity of interactions in the microbial world, emphasizing the sophistication of both bacterial and phage evolutionary strategies.

1.3.1. Bacterial defense mechanisms against phages

Bacterial strategies against phages could be classified based on their external (extracellular) or internal (intracellular) actions within the bacterial cell. To prevent phage attachment externally, bacteria modify their surface receptors using various strategies. These methods range from altering the receptor's structure through mutations, experiencing inheritable changes in receptor expression known as phase variation, to concealing receptors beneath layers such as capsules or extracellular polymers. Additionally, some bacteria create decoy structures aimed at decreasing phage attachment rates (Manning and Kuehn, 2011). Studies reported that several strategies for bacteria against phages from external (Egido et al., 2022): (1) Altering Receptor Structures: Bacteria can mutate in ways that change the shape or composition of their surface receptors, hindering phage binding. Such changes may directly affect the receptor gene or the gene expression control mechanisms. (2) Creating Receptor Masking Proteins: Bacteria could produce proteins that cover phage receptors on their surfaces, making them unreachable for phages. A notable example is the TraT protein in *E. coli*, which attaches to the OmpA protein's exposed regions, blocking phage access. (3) Releasing Outer-membrane Vesicles (OMVs): By emitting OMVs that carry phage receptors, bacteria can mislead phages away from actual cells, thus preventing successful infection. (4) Enhancing Extracellular Matrix Production: Building up an extracellular matrix of polysaccharides, proteins, lipids, and DNA can effectively

obscure phage receptors, preventing phage recognition and attachment. (5) Employing Phase Variation: Bacteria can reversibly alter gene expression through mechanisms like site-specific recombination, slipped-strand mispairing, and epigenetic changes. This phase variation affects bacterial phenotype, including the display of surface proteins such as phage receptors, thereby influencing their availability to phages (Fig. 1.4).

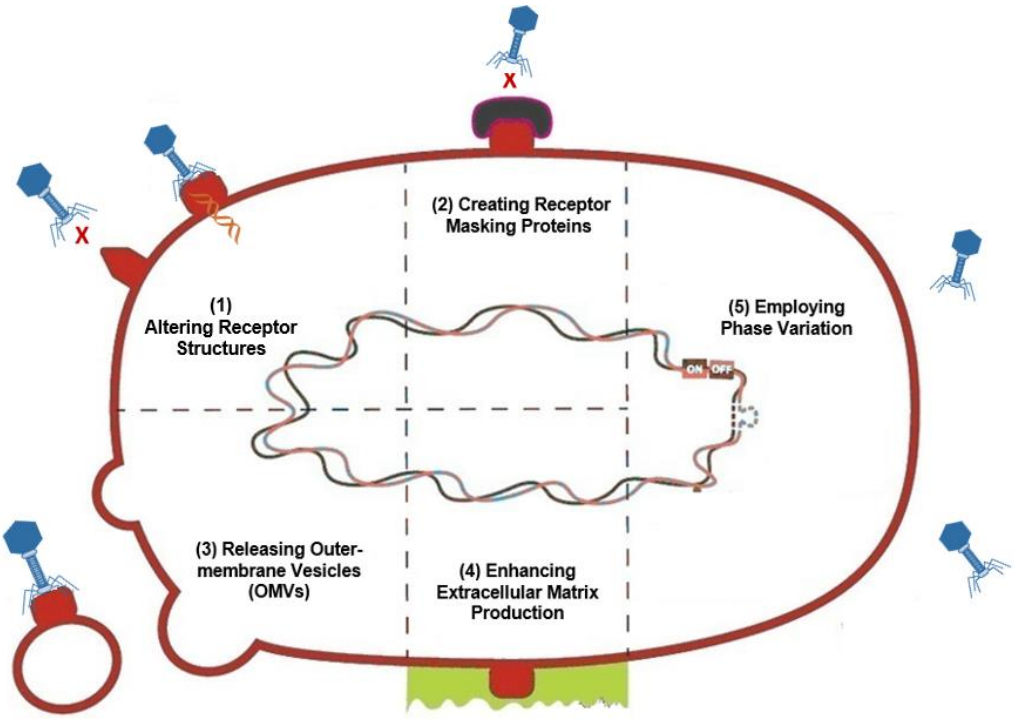


Fig. 1.4. The diagram illustrates studies reporting that bacteria employ several strategies to defend against phages from external (Egido et al., 2022).

During phage invasions, bacteria activate intricate internal defense systems, often located within genomic regions known as defense islands. These areas are hotspots for uncovering novel defense mechanisms against phage attacks. Among these mechanisms are nucleic acid interference strategies, including the Restriction-Modification (R-M) systems, which distinguish between host and foreign DNA through methylation, and modifications to the DNA structure itself. Newer findings like the DISARM (Defense Island System Associated with Restriction-Modification) (Ofir et al., 2018) and BREX (BacteRiophage Exclusion) systems have broadened our

understanding of methylation-based defenses against phages (Gordeeva et al., 2019). In a departure from their eukaryotic counterparts, prokaryotic Argonaute (pAgo) proteins engage in DNA-DNA interference to thwart phage threats. Additionally, the CRISPR-Cas systems represent a unique adaptive immunity mechanism in prokaryotes, allowing bacteria to fend off invasions by mobile genetic elements such as phages and plasmids (Hille et al., 2018). Found across many bacterial genomes and occasionally within plasmids, the CRISPR-Cas system operates through a CRISPR array and a *cas* gene operon. The array consists of spacers derived from foreign DNA sequences, serving as a molecular memory of past invasions, while the *cas* operon encodes Cas proteins, the machinery for the system's immune response. The CRISPR-Cas immunity process unfolds in three phases: adaptation, expression, and interference. During adaptation, foreign DNA fragments are incorporated into the CRISPR array as new spacers. Expression involves the transcription of the CRISPR array into a long precursor RNA, which is then processed into shorter, mature crRNAs. These crRNAs, complexed with Cas proteins, form the effector mechanism that scans for and targets the complementary foreign DNA sequences for cleavage, guided by a protospacer adjacent motif (PAM). CRISPR-Cas systems exhibit diversity in their mechanisms and are categorized into two classes, six types, and 33 subtypes. Class 1 systems feature a multi-subunit Cas complex, while Class 2 systems utilize a single-subunit effector protein for the recognition and cleavage of foreign DNA or RNA. Among these, the Type II CRISPR-Cas system is notably recognized for its application in genome editing technologies. Overall, the CRISPR-Cas systems illustrate the sophisticated strategies bacteria employ to detect, target, and neutralize phage threats, highlighting a key aspect of the microbial defense arsenal against phage predation. To counteract phage proliferation, some bacteria resort to abortive infection (Abi) tactics, sacrificing themselves via toxin-antitoxin mechanisms (Blower et al., 2012a). Bacterial chemical defenses also come into play, involving the production of antimicrobial secondary

metabolites. Recent bioinformatics research has revealed systems like Abi and prokaryotic viperins, which obstruct viral replication through mechanisms that are still being explored. Together, these diverse bacterial defense strategies, ranging from direct nucleic acid interference to the ultimate sacrifice and chemical countermeasures, highlight the dynamic and ongoing evolutionary conflict between bacteria and phages.

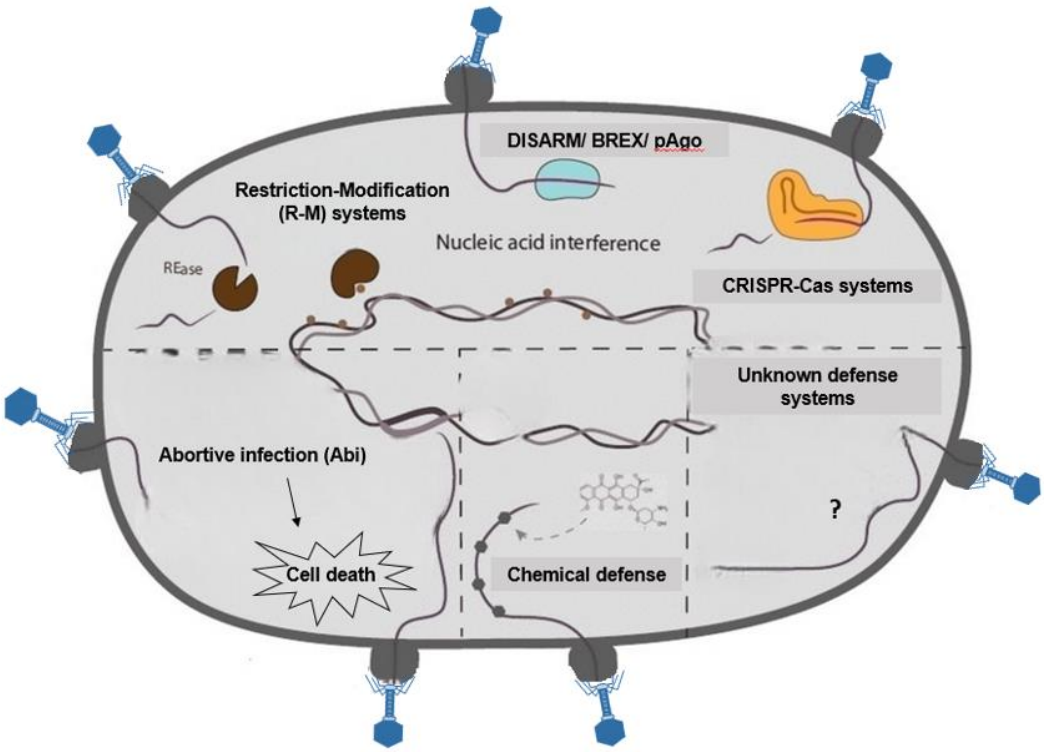


Fig. 1.5. The diagram illustrates studies reporting that bacteria activate complex internal defense systems during phage invasions (Egido et al., 2022).

Phages also can equip bacteria with defense mechanisms against subsequent infections by identical or related phages, a phenomenon known as superinfection exclusion (Sie) (Folimonova, 2012). This includes producing proteins that hide cell surface receptors to block new infections, thereby also safeguarding newly formed phages from being neutralized by receptor remnants from lysed bacterial cells. An example is the T5 phage, which secretes the lipoprotein Llp to obscure its receptor, the outer membrane protein FhuA. Moreover, prophages (phages integrated into the bacterial genome) can contribute to resistance against phage invasion through proteins

that either anchor to or associate with the membrane, thwarting phage DNA entry into the bacterial cytoplasm. These proteins can prevent the formation of channels for DNA entry, inhibit phage lysozyme action on bacterial cell walls, or alter the conformation of proteins at the DNA injection site to block translocation. Beyond Sie mechanisms, prophages also enable resistance via other routes. For instance, the RexA-RexB system in λ-lysogenic *E. coli* reduces the cell's membrane potential, decreasing ATP production and leading to cell death. Phage *Panchino* endows *M. smegmatis* lysogens with a broad-spectrum single subunit R-M system. Additionally, prophages may carry genes for repressor proteins that bind phage DNA, helping safeguard lysogenized bacteria by mitigating inadvertent prophage transcription. Prophages can also induce phenotypic changes in bacteria through genetic elements known as morons, which can express independently of prophage activation. Recent research into *Enterobacteria* P4- and P2-like prophages has uncovered genetic clusters rich in bacterial immune mechanisms, including the phage anti-restriction-induced system (PARIS) (Deep et al., 2024). PARIS induces an abortive infection response upon detecting a phage-encoded anti-restriction protein, Ocr, which targets R-M systems and BREX. The functions of PARIS and other newly identified systems are yet to be fully elucidated. Overall, phages can endow their bacterial hosts with defense strategies against further phage attacks, benefiting both the bacteria and the infecting phage by ensuring the survival and propagation of both.

1.3.2. Counterattack of phages on bacterial defense system

While bacteria showcase a vast array of defense mechanisms against phage infections, phages counter with equally diverse strategies to overcome these defenses. This dynamic interplay demonstrates an evolutionary arms race between bacterial defenses and phage countermeasures. Phages can adapt their receptor-binding proteins (RBPs) through mutations, often facilitated by diversity-generating retroelements (DGRs), to change their cell surface receptor affinity, allowing them to bypass bacterial

surface modifications (Guo et al., 2014). Some phages produce proteins to mask cell surface receptors or use enzymes like depolymerases to penetrate bacterial capsules and extracellular layers. To evade restriction-modification (RM) systems, phages employ several tactics: (1) Avoiding or altering restriction sites in their genome. (2) Modifying sequences recognized by restriction endonucleases. (3) Occluding restriction sites with proteins. (4) Sequestering restriction endonucleases with decoy proteins. (5) Acquiring or inducing methylation of their DNA to mimic host DNA (Egido et al., 2022). For against CRISPR-Cas systems, phages can: (1) Mutate or delete protospacer adjacent motifs (PAMs) or sequences near them to avoid recognition. (2) Deploy anti-CRISPR (Acr) proteins to inhibit CRISPR-Cas activity. (3) Glycosylate their DNA to evade detection. (4) Encase their DNA within a protein shell, shielding it from CRISPR-Cas complexes (Mendoza et al., 2020).

Phages can also outmaneuver abortive infection (Abi) mechanisms by inhibiting toxin-antitoxin system proteases, mutating genes to avoid nucleic acid metabolism-related toxins, or activating their own antitoxin analogs (Blower et al., 2012b). For instance, some phages overcome PLE (Phage-inducible chromosomal island-Like Element) - mediated Abi in *Vibrio cholera*e through phage-encoded CRISPR-Cas systems targeting the PLE genome or endonucleases cleaving PLE replication origins (McKitterick and Seed, 2018). Recent discoveries include phages inactivating the BREX system by targeting its components, like the Ocr protein of phage T7 inhibiting the BREX system's methyltransferase BrxX (Isaev et al., 2020). As research progresses, more phage strategies against bacterial defenses are likely to be uncovered, highlighting the ongoing complexity of interactions in the microbial world.

1.3.3. The diversity in phage-driven variations of resistance mechanism

Phages act as catalysts for genetic diversity within bacterial populations by promoting the development of resistance strategies. The specificity of interactions

between phages and their host bacteria significantly influences the nature of resistance mechanisms, whether they manifest extracellularly or intracellularly. For example, a study explores the resistance mechanisms of marine cyanobacteria, specifically *Synechococcus* and *Prochlorococcus*, against lytic viruses known as cyanophages (Zborowsky and Lindell, 2019). The research differentiates between the cyanobacteria's modes of resistance against specialist and generalist cyanophages within the T7-like and T4-like families. Resistance to specialist cyanophages is predominantly extracellular, preventing the phages from entering the cell. Conversely, resistance against generalist T4-like cyanophages is mostly intracellular, with the cyanobacteria allowing the phage entry but halting its replication at various stages within the cell. Specialist cyanophages failed to attach to most resistant cyanobacterial strains, indicating extracellular resistance. On the other hand, generalist cyanophages attached to nearly all tested resistant strains, signifying intracellular resistance mechanisms were at play. The stage of the intracellular arrest of the infection cycle varied across interactions, indicating multiple, possibly unknown, mechanisms of intracellular resistance within the cyanobacteria. The entry of generalist phages into non-host cyanobacterial cells and the partial replication of their genomes suggest that horizontal gene transfer might extend beyond direct host-phage interactions, potentially influencing the genetic diversity and evolution of marine cyanobacteria. Some strains of *Synechococcus* and *Prochlorococcu*s were found to be polyploid, which might allow them to tolerate partial genome degradation by phages without affecting their growth, suggesting a potential advantage of polyploidy in bacterial resistance. Overall, it suggests that marine cyanobacteria employ diverse strategies to survive phage predation, including mechanisms that allow them to tolerate or prevent the replication of phage genomes. On the other hand, advancements in high-throughput genetic technologies have facilitated detailed examinations of the host genetic factors that confer resistance to a wide array of phages. These studies have illuminated both specific

and general resistance factors, illustrating the elaborate interaction dynamics between phages and their bacterial hosts. Through these interactions, diverse cellular mechanisms for host responses to phage threats are revealed (Mutalik et al., 2020). In *Staphylococcus aureus*, resistance to phages can stem from both enduring genetic modifications and temporary, adaptive changes. This dual nature of resistance mechanisms underlines the challenge in developing effective phage therapy strategies. Specifically, it highlights the necessity for designing phage cocktails capable of navigating or leveraging these resistance strategies to ensure therapeutic efficacy (Jurado et al., 2022). Finally, the evolving understanding of phage-driven variations in resistance mechanisms underscores the complex ecological and evolutionary pressures shaping the interactions between phages and their bacterial hosts. This knowledge not only enhances our grasp of microbial ecology but also informs the development of phage-based applications, including phage therapy, by illuminating potential resistance hurdles and strategies to overcome them.

1.3.4. Multiple factors involved in the development of phage-resistant strains

The selection of phage-sensitive and phage-resistant bacterial strains is influenced by a complex interplay of biological, ecological, and genetic factors. By considering these factors, researchers and clinicians can better predict and manage the dynamics of phage-bacteria interactions, optimize phage therapy, and mitigate the development of phage resistance. (1) Multiplicity of Infection (MOI), the ratio of phages to bacteria, significantly affects the selection pressure on bacterial populations. High MOI can lead to rapid killing of sensitive strains, potentially selecting for resistant mutants. Conversely, a low MOI may allow sensitive strains to grow, but could also promote the emergence of resistance through genetic mutations or horizontal gene transfer. One study reported a mathematical model to simulate the dynamics of phage-host interactions and the development of resistance. The model helped to confirm observations from the strain isolations: in conditions of low MOI, phage-sensitive

strains were dominant in the regrowing population (over 99.8%), whereas phage-resistant strains dominated (over 87.8%) when the initial MOI was high (Christiansen et al., 2016). (2) Bacterial Population Density and Heterogeneity: Dense and genetically diverse bacterial populations can harbor or quickly develop phage resistance through various mechanisms, including spontaneous mutations, phase variation, and CRISPR-Cas systems. The complexity of the bacterial community can thus influence the selection dynamics of sensitive and resistant strains. (3) Phage Adaptability and Diversity: Phages can evolve to overcome bacterial resistance, creating a continuous co-evolutionary arms race. The genetic diversity and adaptability of phages determine their ability to infect new or resistant bacterial strains, influencing the selection process. (4) Environmental Conditions: Factors such as temperature, pH, salinity, and the presence of organic matter can affect phage viability and activity, as well as bacterial growth and defense mechanisms. Environmental conditions thus play a crucial role in the interaction dynamics between phages and bacteria. (5) Phage Treatment Time and Frequency: The timing and frequency of phage applications can impact the development of resistance. Continuous or repeated exposure to phages can select for resistant strains, whereas strategic timing might reduce resistance development. (6) Genetic Exchange and Horizontal Gene Transfer: Bacteria can acquire phage resistance genes through horizontal gene transfer from other bacteria, including conjugation, transformation, and transduction. The genetic plasticity of bacterial populations influences their ability to adapt to phage predation. (7) Fitness Costs of Resistance: Developing resistance to phages often comes with fitness costs for bacteria, such as reduced growth rate or virulence. The balance between the benefits of resistance and the associated fitness costs affects the prevalence of resistant strains. (8) Trade-offs and pleiotropy: The evolution of resistance to phages or antibiotics can involve trade-offs and pleiotropic effects, where resistance to one agent may influence susceptibility to another. These dynamics underscore the complexity of resistance evolution in bacterial

populations (Burmeister et al., 2020). (9) Antibiotic and Phage Synergy: The interaction between antibiotics and bacteriophages is a critical factor in the selection of phage-sensitive and phage-resistant *E. coli* strains. A study demonstrated that combining phages with antibiotics like ciprofloxacin inhibited the growth of *E. coli* and prevented the emergence of resistant mutants. This suggests that phage therapy could be strategically used in conjunction with antibiotics to enhance therapeutic outcomes and manage resistance (Dalmasso et al., 2016).

1.3.5. Strategies to overcome phage resistance

To address phage resistance, researchers have explored a variety of innovative strategies, each with its unique mechanism and application potential. Multiple approaches have been identified to overcome phage resistance, such as deploying phage cocktails, utilizing flavonoids, applying enzymes from phages known as endolysins, genetically modifying phages, and leveraging the coevolutionary adaptation of phages. Each of these strategies offers promise in overcoming phage resistance, with ongoing research aimed at refining their effectiveness and exploring their clinical applications. The choice of strategy may depend on the specific bacterial target, the type of infection, and the environment in which the biocontrol is applied.

Phage cocktails consist of multiple bacteriophages selected for their synergistic or complementary action against a broad range of bacterial strains or to prevent the development of resistance. This approach combines multiple phages into a single treatment, targeting a broader range of bacterial strains and reducing the chance of resistance. If a bacterium becomes resistant to one phage, others in the cocktail can still be effective. This approach also increases the spectrum of activity against diverse bacterial populations. For instance, in combating resistant plant bacterial pathogens, phage cocktails offer a biocontrol strategy that preserves the environmental and human health while addressing bacterial resistance (Farooq et al., 2022). Similarly, phage

cocktails have shown efficacy in preventing the development of phage-resistant strains in *Escherichia coli* by targeting multiple host receptors (Yu et al., 2017).

Flavonoids, natural compounds found in many plants, have been identified for their potential to synergize with phages. They can inhibit bacterial defense mechanisms against phages or directly affect bacterial virulence and biofilm formation, making bacteria more susceptible to phage attack. Flavonoids can also modulate the immune response, potentially enhancing the efficacy of phage therapy (Cushnie and Lamb, 2005). The relationship between the structural configuration of flavonoids and their antibacterial potency has been a significant focus of research, demonstrating how specific structural elements of flavonoids enhance their antimicrobial capabilities (Kumar and Pandey, 2013).

Endolysins are enzymes produced by phages to lyse bacterial cells from the inside at the end of the phage replication cycle. Applying endolysins externally allows for targeted bacterial destruction without the need for phage infection, bypassing traditional resistance mechanisms. Recognized for their role as novel antibacterial agents against foodborne pathogens, endolysins are peptidoglycan hydrolases, encoded by bacteriophages, that dismantle bacterial cells by attacking the cell wall. This action is particularly effective in gram-positive bacteria, where the peptidoglycan layer is more accessible. The specificity, operational mechanism, capacity for modification, and absence of resistance development associated with endolysins have piqued scientific interest in recent times. Advances in employing endolysins for enhancing food safety have been noteworthy. They can eradicate bacterial biofilms linked to foodborne pathogens, and their cell wall-binding domain offers a rapid method for detecting such pathogens in food products (Khan et al., 2023).

Genetic and chemical modifications of phages enhance their bactericidal efficiency and expand their host range. This includes expressing toxin proteins,

modifying host recognition receptors, and crosslinking phage coats with antimicrobial peptides (Guo et al., 2021). The key tactics include modifying the phages' host range by changing or replacing the receptor-binding proteins on their surface, which broadens or alters the spectrum of bacteria they can infect. This aids in specifically addressing bacterial strains resistant to current phage treatments. Additionally, expressing antibacterial enzymes, phages are engineered to possess genes for antibacterial enzymes like endolysins, enabling them to break down bacterial cells both from within and externally, thereby enhancing their killing efficiency. Furthermore, genetic disruption, phages can be engineered to carry genetic elements, such as CRISPR-Cas systems, that specifically target and disrupt bacterial resistance genes, making the bacteria susceptible to phage attack.

The evolution of phages through co-adaptation with their bacterial hosts can lead to the development of phages capable of overcoming bacterial resistance mechanisms. For example, a study demonstrated that a "trained" generalist phage, adapted through prolonged coevolution with its host, suppressed bacterial growth more effectively than non-adapted phages or phage cocktails (Borin et al., 2022). Similarly, a study demonstrated that a phage, identified as λtrn, which had undergone a 28-day training period, significantly outperformed its untrained counterpart in controlling *E. coli* B strain REL606 proliferation in vitro (Borin et al., 2021). Additionally, another study utilizes a model system comprising bacteriophage λ and *E. coli* to examine their swift coevolutionary interaction under controlled laboratory conditions. This evolutionary interplay is marked initially by *E. coli* developing resistance through alterations in the malT gene, which impacts the expression of the LamB receptor. In response, the λ phage adapts by modifying the binding domain of its host recognition protein J, allowing it to target an alternative receptor, OmpF. (Gupta et al., 2022). These studies highlight the advantages of undergoing phage training. These advantages encompass

extended bacterial population control, a slowdown in the evolution of bacterial resistance, and heightened effectiveness in killing bacteria.

The combination of phages with antibiotics is another promising approach to constrain bacterial resistance evolution. Phage-antibiotic combinations can suppress or even reverse bacterial resistance to antibiotics, offering a synergistic effect that enhances the efficacy of bacterial infection control (North and Brown, 2020). The concept of phage–antibiotic synergy (PAS) was introduced by Comeau et al., who observed an enhanced effect when bacteriophages were combined with sublethal doses of β-lactam antibiotics. This combination led to an increase in phage burst size, which refers to the number of phages released from a host bacterial cell following lysis. The researchers linked the PAS effect mainly to the phenomenon of antibiotic-induced cellular filamentation. In this altered state, bacterial cells continue to elongate without undergoing division. This process is believed to facilitate increased production of components necessary for phage assembly, thereby enhancing the efficiency of phage replication within the filamenting bacterial cells (Comeau et al., 2007).

1.4. Research objectives

This research presents a strategic method aimed at improving the effectiveness of bacteriophages in combating the foodborne pathogenic *E. coli* O157:H7, with an emphasis on understanding the genetic basis of resistance to phages within *E. coli* populations.

The primary goals of this research are:

a) To evaluate the effects of candidate genes on the phage-resistance of *E. coli* populations through single gene-deletion strains from Keio collection model.

b) To identify substances that can inhibit the functions of candidate genes and examine combined effects of phages with these substances to improve the lytic activity of phages against *E. coli* O157:H7.

c) To explore synergistic strategies for overcoming the regrowth of phage-resistant *E. coli* populations, ensuring sustained effectiveness of phage treatments.

Chapter 2. The combined impact of phages and substances that interact with primosomal protein A on *Escherichia coli*

2.1. Introduction

To develop effective microbial control strategies with phages, it is crucial to understand the mechanisms behind phage resistance. This requires a detailed knowledge of the genes that contribute to both resistance and susceptibility to phages, which will aid in the successful elimination of foodborne pathogens through phage application. The Keio collection, which comprises single-gene deletion mutants, serves as a renowned innovative resource for investigating the unidentified biological processes and impacts of genes and their connections in the commonly utilized *Escherichia coli strain* K-12 BW25113 (Baba et al., 2006). The Keio collection has been utilized to pinpoint bacterial genes that influence susceptibility to antibiotics and resistance (Tran et al., 2011); however, there have been no prior attempts to use the Keio collection model to screen for genes necessary for resistance to phages in foodborne bacteria**.** In our preliminary experiment, from the Keio collection, we screened a total of 3,909 *Escherichia coli* BW25113 mutants, each lacking a single gene. Our goal was to identify specific genes that are connected to phage resistance. Our first step was to identify phages that particularly target *E. coli* BW25113. We characterized and utilized Phage S127BCL3, which was isolated from chicken liver and can infect both *E. coli* BW25113 and O157:H7, for this screening process (Fig. 2.3 and 2.4). The genes involved in resistance to phage S127BCL3 were pinpointed by examining deletion mutants that displayed heightened vulnerability to the phage. Increased susceptibility to phage S127BCL3 was observed in ten gene-deletion mutants (*Δrpe, ΔyfcD, ΔybcH, ΔyaiW, Δgor, ΔubiE, ΔdnaK, ΔtolR, ΔpriA,* and *ΔtolA*) as indicated in Fig. 2.5 and 2.6. Notably, the *ΔpriA* gene-deletion mutant demonstrated significant vulnerability to phage S127BCL3. The gene priA, as described by Lee et al. in 1990, encodes a protein called PriA, which functions as a primosomal replicative

protein. This protein is likely crucial for the mechanism of phage resistance, suggesting its significant role in this biological process (Lee et al., 1990).

Numerous theories about bacterial defense mechanisms against phages have been suggested, though many aspects are still unclear. An absence of the phage receptor on the surface of the bacterial cell is one such mechanism that prevents the phage from attaching to the bacteria. By hindering the phage's ability to bind to the bacterial cell, this receptor deficiency effectively blocks the initial step of infection. This deficiency results in the bacteria developing resistance to phages (Hashemolhosseine et al., 1994). Another protective strategy is the CRISPR/Cas system, a sophisticated immune response that cuts out and retains segments of foreign DNA. This mechanism in *E. coli* captures DNA from invaders and incorporates it into CRISPR sites for subsequent immune defense. A study has demonstrated that the CRISPR/Cas system grants bacteria resistance to phage infections (Barrangou et al., 2007). The CRISPR-Cas system functions through two phases: adaptation, which involves the acquisition of DNA, and interference, where it targets and destroys invader DNA. There are two different types of adaptation within the CRISPR-Cas system: primed adaptation and naive adaptation.

Primosomal protein A (PriA) plays a critical role in the CRISPR-Cas system's primed adaptation phase (Ivančić-Baće et al., 2015), essential for resistance against phages. PriA initiates DNA replication at stalled or damaged forks, maintaining genome integrity and linking DNA repair to phage immunity in the CRISPR-Cas adaptation process (Fig. 2.1). This coordination helps bacteria incorporate foreign DNA into the CRISPR array, enhancing defense against similar future phage attacks. In primed adaptation, the CRISPR-Cas system leverages previous encounters with invader DNA to enhance its capability to capture and integrate new DNA segments from the same source. This adaptive response involves proteins like RecG and PriA in *E. coli*, which aid in DNA capture and break down R-loop structures formed during DNA interaction with the Cascade-crRNA complex. This exposes ssDNA, allowing the Cas1-

Cas2 complex to engage and cut the DNA for spacer acquisition, reflecting a strategy that evolves from past interactions (Fineran et al. 2014, Ivančić-Baće et al. 2015). Therefore, blocking PriA's activity could impede the CRISPR-Cas system's ability to acquire new spacers, potentially slowing the development of bacteriophage-resistant bacteria. However, the impact of PriA on phage resistance and vulnerability has not been thoroughly investigated. To elucidate PriA's influence on bacteriophage resistance in *E. coli*, the study in this chapter 2 explores substances reported to inhibit PriA's function for their effects on phage vulnerability in *E. coli* BW25113 (Parent strain) and O157:H7. This investigation aims to provide deeper insights into PriA's role in bacteriophage resistance mechanisms and potentially identify novel approaches to control the spread of resistant bacterial strains.

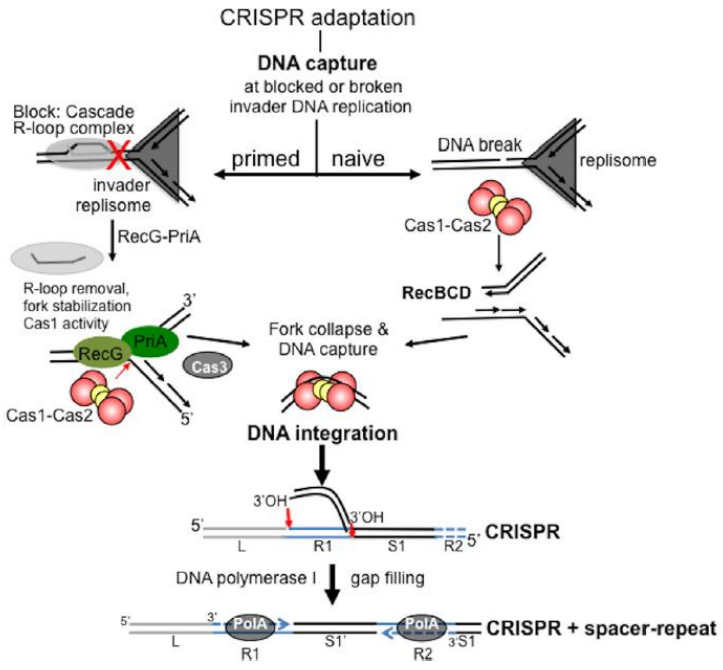


Fig. 2.1. Model suggesting varying needs for DNA capture in primed and naive adaptation in *E. coli.* In primed adaptation, RecG and PriA detect these blockages, where PriA binds to the fork's 3' end, limiting remodeling activities. This is maintained until RecG's helicase activity remodels the forks and clears the R-loops (Ivančić-Baće et al., 2015).

2.2. Materials and methods

2.2.1. Bacterial strains, inoculum preparation, and culture conditions

From the National Institute of Genetics in Shizuoka, Japan, we obtained 3,909 mutants with single-gene deletions (kanamycin-resistant) from the Keio collection, as well as the parental strain *Escherichia coli* BW25113, which is the wild-type and lacks kanamycin resistance. These strains and mutants were used in our research to investigate the genetic factors influencing various bacterial functions and responses. Various bacterial strains were sourced from different institutions: *Salmonella* Typhimurium NBRC12529 came from the Biological Resource Center at the National Institute of Technology and Evaluation in Tokyo, Japan; *Staphylococcus aureus* NCTC8325 was obtained from the UK Health Security Agency in Salisbury, UK; *Listeria monocytogenes* ATCC15313 was purchased from the American Type Culture Collection in the USA; and *Bacillus cereus* JCM2152 was acquired from the Japan Collection of Microorganisms at the RIKEN BioResource Research Center in Ibaraki, Japan. Additionally, multiple strains of *Escherichia coli* O157:H7, including EC-064, EC-027, EC-041, EC-140, EC-080, EC-071, EC-119, EC-138, and EC-140, were sourced from the Fukuoka City Institute of Health and Environment, Japan. These bacterial strains were cultivated in Luria-Bertani (LB) Miller broth from Kanto Chemical in Tokyo, Japan, and on Tryptic Soy Agar (TSA) from Oxoid in the UK. They were stored at -80°C in 15% glycerol to maintain viability over long periods. For experimental use, the strains were precultured overnight at 37°C in 5 mL of LB with shaking at 130 rpm, starting from a single colony picked from a TSA plate. The single gene-deletion mutants required LB and TSA supplemented with 200 mg/L kanamycin to maintain selective pressure for kanamycin resistance. The careful handling and preparation processes guaranteed that all bacterial strains used in the study had consistent and reproducible growth conditions.

2.2.2. Isolation and cultivation of the phage S127BCL3

From chicken livers, phages targeting *E. coli* O157:H7 strains were collected. Out of the 32 isolated phages, those capable of infecting *E. coli* BW25113 were identified using the spot-test screening method. Phage S127BCL3 was specifically selected due to its strong lytic activity against *E. coli* BW25113, as evidenced by tube-based phage vulnerability assays performed at a multiplicity of infection (MOI) of 0.1. To further understand its range, Phage S127BCL3 was tested against other bacterial strains, including *S.* Typhimurium IFO12529, *S. aureus* NCTC8325, *L. monocytogenes* ATCC15313, and *B. cereus* JCM2152. Separately, the well-known T7 phage, part of the Podoviridae family, was acquired from the National BioResource Project in Shizuoka, Japan. For propagation, this phage was combined with *E. coli* BW25113 and cultured overnight at 37°C in LB medium with shaking to ensure exponential growth. After incubation, the mixture was centrifuged at 12,000 × *g* for 5 minutes at 4°C. The supernatant was filtered using a 0.22 µm pore size sterilization filter (As One Corp., Osaka, Japan) to obtain a phage solution. This phage stock was stored at 4°C and had a titer of 10^{10} PFU/mL, determined by the number of plaques formed after overnight incubation. For storage and further experiments, the phage was diluted in a saline magnesium (SM) buffer, which includes 0.05 mol/L Tris-HCl buffer (pH 7.5), 0.1 mol/L NaCl, 0.008 mol/L $MgSO_4$, and 0.01% gelatin. This detailed procedure ensured that all bacterial strains were consistently and reproducibly cultured for the study.

2.2.3. Screening genes for phage resistance

The 3,909 mutants with single-gene deletions from the Keio collection underwent a comprehensive screening for phage resistance. Each mutant was initially adjusted to a bacterial concentration of 10^8 CFU/mL. For the screening process, 10 µL of this bacterial solution was added to each well of a 96-well plate, which contained 180 µL of LB medium supplemented with 1 mmol/L $CaCl_2$. To this mixture, 10 µL of phage S127BCL3 (10^9 PFU/mL) was introduced. The plates were incubated with shaking at 37°C for 48 h, and each experiment was conducted in triplicate (n = 3). Turbidity

measurements, which indicate bacterial growth, were taken at 0, 6, 24, and 48 h. Mutants that showed no increase in turbidity at both 24 and 48 h post-phage addition were identified as potential candidates with enhanced phage resistance. This careful screening process helped identify mutants with altered susceptibility to phage infection, contributing valuable insights into bacterial defense mechanisms against phages. To ensure accuracy and reliability, the bacterial cultures were prepared with precise concentrations, and the LB medium was consistently supplemented with $CaCl_2$ to facilitate phage infection. The triplicate experiments and systematic turbidity measurements provided robust data for identifying phage-resistant mutants.

2.2.4. Phage susceptibility assay

In the experiment with phage treatment, a 500 µL bacterial solution at 10^8 CFU/mL was mixed with 4 mL of LB medium, 1 mmol/L $CaCl_2$, and 500 µL of phage S127BCL3 at a titer of 10^6 PFU/mL, achieving a multiplicity of infection (MOI) of 0.01. The mixture was then incubated with shaking at 37°C and 130 rpm for 48 h, and this process was repeated three times (n = 3). For the control setup, 4 mL of LB medium containing 1 mmol/L $CaCl_2$ was combined with 500 µL of SM buffer and 500 µL of the bacterial solution. This control mixture was also incubated under the same conditions of 37°C with shaking for 48 h. Viable cell counts were taken at intervals of 0, 2, 4, 6, 8, 12, 24, and 48 h. To determine the number of viable cells, 20 µL samples from both the phage-treated and control groups were placed into a 96-well plate with 180 µL of phosphate-buffered saline. Additionally, 10 µL samples from 10-fold serial dilutions were spotted, and 100 µL from the undiluted cultures were spread on TSA plates. Following overnight incubation at 37°C, colonies were counted to calculate the viable bacterial counts. The experiment also included the well-known T7 phage to determine if the observed increase in phage sensitivity from gene deletion in *E. coli* was specific to phage S127BCL3 or a broader range applicable to other *E. coli* phages.

2.2.5. Choosing and preparing substances that interact with PriA

Based on earlier studies (Huang et al., 2015; Voter et al., 2018), kanamycin, chloramphenicol, and kaempferol were selected due to their known inhibition of PriA function. To determine the minimum inhibitory concentration (MIC) of these substances against *E. coli* BW25113 without the presence of phages, the broth dilution method was utilized. This involved culturing the bacteria in 5 mL of LB medium at 37°C with shaking at 130 rpm for 24 h. Kanamycin monosulfate (Nacalai Tesque, Inc., Japan) was prepared by dissolving it in pure water to create a 20 mg/mL solution, which was then filtered through a 0.22 µm sterile membrane filter (As One, Corp., Osaka, Japan). The solution was subsequently diluted to final concentrations of 200 µmol/L and 500 µmol/L. Chloramphenicol (Nacalai Tesque, Inc., Japan) was prepared in 99.5% ethanol to make a 20 mg/mL solution, filtered, and diluted to achieve concentrations of 200 µmol/L and 500 µmol/L. For kaempferol (Tokyo Chemical Industry, Corp., Japan), it was dissolved in dimethyl sulfoxide to form a 28.6 mg/mL solution, filtered, and then diluted to final concentrations of 50 mmol/L and 100 mmol/L. For control purposes, tetracycline hydrochloride (Nacalai Tesque, Inc., Japan) was used. It was dissolved in pure water at a concentration of 20 mg/mL, filtered, and diluted to final concentrations of 50 µmol/L and 100 µmol/L. This setup ensured that the substances were tested under consistent conditions to accurately determine their MIC values against *E. coli* BW25113.

2.2.6. Treatment with phage S127BCL3 and PriA inhibitors on *E. coli* strains

To evaluate the impact of phage S127BCL3 and PriA-interacting substances on *E. coli* BW25113 and O157:H7 (EC-071), a series of controlled experiments were conducted. For the treatment of *E. coli* BW25113, a 500 µL suspension of the bacteria (10^8 CFU/mL) was added to 4 mL of LB medium with 1 mmol/L $CaCl_2$, along with 50 µL of the substance solution and 500 µL of the S127BCL3 phage solution (10^5 PFU/mL, MOI=0.001, diluted in SM buffer). This mixture was incubated at 37°C with shaking for 24 h. For *E. coli* O157:H7, the experimental setup was similar, but the phage

solution concentration was adjusted to 10^6 PFU/mL (MOI=0.01, diluted in SM buffer). Control groups were prepared by replacing the substance and phage solutions with solvents and SM buffer. Viable cell counts were recorded at intervals of 0, 3, 6, 9, 12, and 24 h to monitor bacterial growth and phage efficacy. Tetracycline served as a negative control, acting as a protein synthesis inhibitor that does not interact with PriA, unlike kanamycin and chloramphenicol. The final concentrations for the substances were as follows: kanamycin: 2 μmol/L and 5 μmol/L, chloramphenicol: 2 μmol/L and 5 μmol/L, kaempferol: 0.5 mmol/L and 1 mmol/L, tetracycline: 0.5 μmol/L and 1 μmol/L. To prevent precipitation of kaempferol, half-strength LB with 1 mmol/L $CaCl_2$ was used instead of full-strength LB with $CaCl_2$ during kaempferol treatments. This thorough protocol allowed for a detailed assessment of the combined effects of phage and PriA-interacting substances on *E. coli* strains, providing valuable insights into potential resistance mechanisms and interactions.

2.2.7. Evaluating *priA* gene transcription in *E. coli* post-phage exposure

2.2.7.1. Bacterial cell exposure to phage

To investigate how phage exposure influences *priA* gene transcription in *E. coli*, overnight cultures of the BW25113 and O157:H7 strains were grown at 37°C in 5 mL of LB medium with shaking at 130 rpm. For the phage-treated group, 500 μL of the bacterial suspension (10^8 CFU/mL) was combined with 4 mL of LB medium supplemented with 1 mmol/L $CaCl_2$. Afterward, 500 μL of a phage solution, adjusted to 10^5 PFU/mL (MOI=0.001), was added and mixed thoroughly. In the control setup, 500 μL of the bacterial suspension and 500 μL of SM buffer were added to 4 mL of LB medium containing 1 mmol/L $CaCl_2$, omitting the phage solution. Both the phage-treated and control samples were then incubated at 37°C with shaking at 130 rpm for 16 h. This design provided a basis for comparing the phage-treated and untreated *E. coli* cultures, enabling an evaluation of the effects of phage exposure on bacterial growth and *priA* gene transcription under controlled conditions.

2.2.7.2. Post-cultivation processing and RNA extraction

After cultivation, the bacterial samples were placed into 5.0 mL tubes. These were then centrifuged at 9,000 × *g* for 5 minutes to pellet the cells. The supernatant was carefully removed by suction. The cell pellets were resuspended in 0.6 mL of Buffer RLT from the RNeasy Mini Kit (QIAGEN, Germany) and lysed by vortexing for 1 minute. Next, 0.35 mL of 70% ethanol was added to the lysates, and the mixture was thoroughly homogenized by pipetting. The RNA extraction was performed using the RNeasy Mini Kit according to the manufacturer's instructions. The concentration of the total RNA extracted was determined with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). To assess the quality of the RNA, an Agilent 2100 bioanalyzer electrophoresis system (Agilent, USA) was used, ensuring the RNA met the required standards for subsequent analyses. This detailed protocol ensured the isolation of high-quality RNA from bacterial cells, which is crucial for reliable downstream applications such as qPCR, RNA sequencing, and other molecular biology techniques.

2.2.7.3. cDNA synthesis

To generate cDNA, RNA was subjected to reverse transcription, where total RNA served as the template for synthesizing cDNA. This process was carried out using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan), following the instructions provided by the manufacturer.

2.2.7.4 Quantitative polymerase chain reaction (qPCR)

Quantitative PCR (qPCR) was employed to measure the transcription level of the *priA* gene. Specific primers for the priA gene and a segment of the 16S rRNA gene were designed using Clone Manager Suite 7 software (© 2020 Sci Ed Software LLC). These primers were synthesized by Eurofins Genomics, Japan Co., Ltd. The 16S rRNA primers served as housekeeping genes for normalization. The primer details are provided in Table 2.1. To provide evidence for proof of the specificity of the primers

designed for the RT-qPCR, the specificity of the primers was examined by PCR using genomic DNA extracted from *E. coli* BW25113 or O157:H7 as a template.

The molecular sizes of the PCR products obtained by both designed primers were confirmed by gel electrophoresis, the PCR reaction mixture had a total volume of 10 µL and consisted of: 5 µL of 2X GoTa q® Green Master Mixes (Promega, Japan), 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 1 µL of 489 ng/µL genomic DNA extraction from host strain, and 2 µL of nuclease-free water. Sterilized Milli-Q water was used instead of DNA for the control group in PCR. The PCR protocol included an initial denaturation step at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 30 seconds. Subsequently, a final elongation 72°C for 5 minute was followed.

The qPCR reaction mixture had a total volume of 20 µL and consisted of: 10 µL of THUNDERBIRD® SYBR qPCR Mix (TOYOBO, Japan), 0.04 µL of 50 × ROX reference dye, 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, 1 µL of 800 ng/µL cDNA, and 7.96 µL of nuclease-free water. Sterilized Milli-Q water was used instead of cDNA for the control group in qPCR. The qPCR protocol included an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 30 seconds. Subsequently, a denaturation at 95°C for 1 minute was followed by annealing at 55°C for 30 seconds, and a final denaturation at 95°C for 30 seconds. The qPCR reactions were carried out on the MX 3000P Real-Time PCR System (Stratagene, California, USA). Results were analyzed with MxPro™ Software version 3.00 (Stratagene, California, USA). RNA quantity was determined based on the Ct value derived from the cDNA synthesized during reverse transcription, and the results were calculated using the comparative Ct method ($2^{-\Delta Ct}$ method).

2.2.8. Statistical evaluation

The experiments were conducted three times for each condition to ensure reliability and reproducibility of the results. The data are presented as mean values accompanied by standard deviations to reflect variability. For statistical analysis, a one-way analysis of variance (ANOVA) followed by a *t*-Test was conducted using Microsoft Excel 2016 for Windows to determine significant differences between the treatment and control groups. A *p*-value of less than 0.05 was considered indicative of statistical significance. This rigorous approach ensured that the findings were robust and that any observed differences were unlikely to be due to random chance.

2.3. Results

2.3.1 Identification and characterization of phage S127BCL3

The objective of this research was to find phages capable of lysing *E. coli* BW25113 using single-gene knockout mutants of non-pathogenic *E. coli* BW25113 as host cells. Initially, thirty-two phages were isolated from raw meat samples. These phages were then screened for their ability to lyse *E. coli* BW25113, and fourteen were identified as having lytic activity. Among the fourteen phages, phage S127BCL3 was particularly noteworthy due to its strong lytic action against *E. coli* BW25113. Notably, this phage did not exhibit any lytic activity against other bacterial species that were tested, including *S.* Typhimurium, *S. aureus, L. monocytogenes,* and *B. cereus*. This specificity underscores the potential of phage S127BCL3 as a targeted agent against *E. coli* BW25113, highlighting its effectiveness and specificity in bacterial lysis (as detailed in Table 2.2). After propagation, the titer of phage S127BCL3 reached approximately 10^{10} PFU/mL. Electron micrographs displayed in Fig. 2.3 revealed that phage S127BCL3 has a head diameter of 78.0 ± 8.2 nm and a tail length of 86.1 ± 4.1 nm, with a contractile tail indicating its classification within the *Myoviridae* family. *Myoviridae*, a family previously recognized under the order *Caudovirales*, has been abolished in the recent updates by the International Committee on Taxonomy of Viruses (ICTV). The members of *Myoviridae* have now been reclassified into several new

families within the class *Caudoviricetes* based on genomic characteristics rather than morphology. The genome of phage S127BCL3 spans 135,530 base pairs, containing 215 open reading frames (ORFs) and a GC content of 43.2%. The nucleotide sequence has been registered under accession number OQ626344 in the GenBank. The genome map, provided in Fig. 2.4, indicates the presence of genes for two lytic enzymes and the absence of genes associated with lysogenicity. Additionally, ten genes are predicted to encode tail fiber proteins, with five of these sharing over 97% sequence identity with those of *E. coli* phage vB_EcoM-ECP26. Therefore, based on genome similarity, phage S127 BCL3 could be reclassified into the *Vequintavirinae* family, similar to phage vB_EcoM-ECP26. This comprehensive analysis underscores the specificity and potent lytic capability of phage S127BCL3 against *E. coli* BW25113, along with detailed genetic and structural characterization, highlighting its potential utility in phage therapy and bacterial research.

2.3.2 Screening and susceptibility testing of *E. coli* BW25113 and mutants

The research began with a comprehensive screening of 3,909 single-gene knockout mutants of the non-pathogenic *E. coli* BW25113 strain, conducted in 96-well plates. Among these mutants, ten (*Δrpe, ΔyfcD, ΔybcH, ΔyaiW, Δgor, ΔubiE;*JW5581, *ΔdnaK, ΔtolR*8*, ΔpriA, and ΔtolA*) exhibited significantly lower turbidity than the wild-type strain after 48 h of incubation at a multiplicity of infection (MOI) of 10. This reduced turbidity indicated that these mutants were potentially more susceptible to phage infection, as illustrated in Fig. 2.5. In parallel, the wild-type parental strain *E. coli* BW25113 was tested for phage susceptibility. Initial observations showed no change in turbidity after 6 h of incubation with the phage. However, after 48 h, the turbidity increased to levels similar to those of the wild-type strain incubated without the phage, suggesting the presence of a phage-resistant cell population within the *E. coli* BW25113 culture. Following the initial screenings, a detailed phage susceptibility assay was conducted to further evaluate the ten identified mutants. This involved

monitoring the viable cell counts of both the mutants and the wild-type strain over a 48-hour period in the presence of the phage. The results from this assay revealed that the *ΔpriA* mutant displayed significantly higher vulnerability to phage S127BCL3 compared to the other nine mutants. This heightened susceptibility is detailed in Fig. 2.6. The findings from these experiments underscore the critical role of the priA gene in conferring resistance to phage infection in *E. coli* BW25113. The increased susceptibility of the *ΔpriA* mutant to phage S127BCL3 suggests that targeting the *priA* gene could be a promising strategy to enhance the efficacy of phage therapy. These results pave the way for further research into the genetic mechanisms of phage resistance and the development of targeted phage therapy approaches to combat bacterial infections. To investigate the impact of phage treatment on the *ΔpriA* mutant strain, the bacterial cultures were exposed to phage S127BCL3 at different multiplicities of infection (MOIs). At an MOI of 10, a substantial reduction in viable bacterial counts was observed in the ΔpriA mutant strain, with numbers dropping to approximately $10^{1.5}$ CFU/mL after 4 h of incubation. This reduction was significantly more pronounced compared to the wild-type BW25113 strain, which maintained higher viable counts under the same conditions. Further analysis showed that after 12 h of incubation, the viable bacterial counts in the *ΔpriA* mutant were about 6 logs lower than those in the phage-treated BW25113 strain. This dramatic reduction was sustained, with the viable counts remaining at approximately 10^6 CFU/mL even after 24 and 48 h of incubation. The study also explored the effects of a lower MOI of 0.01 on the *ΔpriA* mutant strain. Results indicated that after 48 h, the viable cell count for the *ΔpriA* mutant was 1 log lower than that of the wild-type strain, and after 12 h, it was 5 logs lower (Fig. 2.7a). Moreover, the *ΔpriA* mutant exhibited heightened susceptibility to the T7 phage from the *Podoviridae* family, showing greater vulnerability compared to the wild-type strain (Fig. 2.7b). These findings provide comprehensive insights into the genetic factors influencing bacterial resistance to phage infections. The increased phage

susceptibility observed in the *ΔpriA* mutant highlights the crucial role of the *priA* gene in conferring resistance, suggesting that targeting this gene could enhance the effectiveness of phage therapy. The significant reduction in viable bacterial counts in the *ΔpriA* mutant underscores the potential of phage treatments in managing bacterial populations and combating infections.

2.3.3. Exploring the role of PriA in phage resistance

The study aimed to understand the involvement of PriA protein in phage resistance by evaluating the survival of *E. coli* BW25113 when exposed to phage S127BCL3 and various PriA inhibitors. Previous research (Huang et al., 2015; Voter et al., 2018) identified kanamycin, chloramphenicol, and kaempferol as interacting with PriA. Consequently, kanamycin and chloramphenicol, both of which inhibit protein synthesis, were chosen for the experiments, while tetracycline was included as a negative control due to its non-interaction with PriA. The minimum inhibitory concentrations (MIC) established were 5 µmol/L for kanamycin, 100 µmol/L for chloramphenicol, and 10 µmol/L for tetracycline. For treatment purposes, kanamycin and chloramphenicol were used at concentrations of 2 µmol/L and 5 µmol/L, while tetracycline was used at 0.5 µmol/L and 1 µmol/L. Chloramphenicol concentrations were specifically chosen to inhibit PriA function below 40 µmol/L, minimizing bactericidal effects and side effects (Huang et al. 2015). For kaempferol, with an MIC exceeding 512 µg/mL (1.79 mmol/L), treatment concentrations were set at 0.5 and 1 mmol/L (Zhou et al., 2022). As shown in Fig. 2.8d, combining tetracycline with the phage did not prevent the regrowth of resistant bacteria, and there was no notable difference in the viability of *E. coli* BW25113 across the tetracycline concentrations tested with the phage. However, for kanamycin (Fig. 2.8b), a 2 µmol/L concentration did not affect growth, but a reduction was observed at 5 µmol/L. In the presence of the phage, a significant drop of about 5 logs in viable *E. coli* counts was noted within 3 h of incubation, regardless of the kanamycin concentration. When 2 µmol/L of kanamycin was used in combination with

the phage, the regrowth of resistant cells was significantly reduced. Furthermore, combining the phage with 5 µmol/L of kanamycin completely prevented the regrowth of resistant cells. When treated with chloramphenicol alone at a concentration of 5 µmol/L, no notable impact on *E. coli* growth was observed (Fig. 2.8a). The viable *E. coli* counts decreased by 4 logs when exposed to the phage alone. This effect was intensified by an extra log reduction when 5 µmol/L chloramphenicol was used in combination with the phage. Additionally, the combined treatment of chloramphenicol and the phage delayed the regrowth of cells that were resistant to the phage. However, by 24 h, the viable counts had returned to levels similar to those seen in controls treated with the phage alone and with 2 µmol/L chloramphenicol. With both the phage and 5 µmol/L chloramphenicol, the *E. coli* counts stayed 1 log below the control level after 24 h. Conversely, there were no notable differences in the viability of *E. coli* BW25113 when treated with the phage alone or in combination with kaempferol at 0.5 and 1 mmol/L concentrations (Fig. 2.8c). This indicates that kaempferol does not significantly enhance phage activity under the tested conditions. These results highlight the potential of using kanamycin and chloramphenicol in combination with phages to suppress the growth of phage-resistant *E. coli* populations, emphasizing the critical role of PriA in phage resistance mechanisms.

2.3.4. Impacts of phage and PriA inhibitors on *E. coli* O157:H7

The impact of combining phage with kanamycin or chloramphenicol, previously shown to inhibit phage-resistant *E. coli* BW25113, was also examined on *E. coli* O157:H7. Using phage S127BCL3 with 5 µmol/L kanamycin significantly delayed *E. coli* O157:H7 regrowth, resulting in a 2-log reduction in bacterial count compared to the control after 24 h (Fig. 2.9b). Notably, this concentration of kanamycin alone had no effect on bacterial growth. Similarly, combining chloramphenicol with the phage produced comparable inhibitory effects on *E. coli* O157:H7 (Fig. 2.9a). These findings

highlight the potential for using phage in combination with antibiotics like kanamycin and chloramphenicol to control pathogenic *E. coli* O157:H7.

2.3.5. Alterations in *priA* gene expression in *E. coli* BW25113 and O157:H7 following phage treatment

Firstly, the specificity of the designed primers used for RT-qPCR was examined by PCR by the extracted *E. coli* DNA as template. The molecular sizes of the PCR products obtained by both designed primers were confirmed by gel electrophoresis, showing the single band on the targeted size of 125 bp for the *priA* gene and 97 bp for the *16S rRNA*, respectively, without non-specific bands Fig 2.2. Secondly, to investigate changes in *priA* gene expression following phage treatment, after 16 h of treatment, samples were taken from *E. coli* cells both exposed to and not exposed to phage. The relative transcription levels of the *priA* gene in *E. coli* BW25113 and O157:H7 were then measured using qPCR. Figure 2.10 illustrates these transcription levels for cells with and without phage treatment. In both strains, the transcription level of *priA* increased significantly, by more than 16-fold, following 16 h of phage exposure compared to the control group that did not receive phage treatment. Finally, the significant upregulation of *priA* expression following phage treatment suggests a critical role for this gene in the bacterial response to phage infection, offering further insights into bacterial defense mechanisms.

2.4. Discussion

There are two switch modes of chromosomal DNA replication found in the *Escherichia coli.* Therefore, *E. coli* utilizes two priming reactions for DNA replication: the normal OriC system and the phiX system, which is named after phage ΦX174 and requires a more complex primosome for initiation (Masai et al., 1994). Masai et al. (1994) noted that the OriC replication system is dependent on the DnaA protein. However, they also described an alternative replication system that utilizes the PriA-based primosome. According to Masai and Arai (1996), *E. coli* has the ability to switch

between these two distinct replication modes in response to varying environmental conditions, demonstrating a remarkable adaptability (Masai and Arai, 1996). Typically, chromosome replication is carried out by the high-fidelity DnaA-dependent method; However, under adverse conditions such as DNA damage or nutrient scarcity, the bacteria may temporarily adopt the lower fidelity PriA-dependent mode associated with recombination-dependent DNA repair. When infected by phages, *E. coli* cells, which are already under stress, might thus predominantly rely on PriA for DNA replication. PriA is one of seven key primosomal proteins, alongside PriB, PriC, DnaT, DnaB, DnaC, and DnaG (Sandler and Marians, 2000). It is crucial for the primosome's proper positioning to remove single-strand binding proteins (SSBs) from single-stranded DNA (ssDNA). PriA consists of two main domains: an N-terminal replication fork recognition domain and a C-terminal DNA helicase domain. Within the N-terminal domain, there is a specialized pocket structure that binds to the 3' end of the DNA strand. This structure allows PriA to identify and attach to the arrested replication fork, facilitating the initiation of DNA replication by positioning the primosome correctly (Michel and Sandler, 2017). Deletion of the *priA* gene results in inhibited DNA replication mechanisms in *priA*-deficient strains compared to wild-type strains, based on the aforementioned findings. After inserting their own phage DNA into the host bacterium, phages increase the number of daughter phages by utilizing the host bacterium's DNA replication mechanism, which are then released from the host bacterium by lysis. Therefore, it is assumed that if the DNA replication mechanism of the host bacterium is inhibited, the phage's bactericidal effect would diminish. However, the results of this study show that the *priA*-deficient strain was more susceptible to phage than the wild-type strain. Since phage treatment effectively killed the *priA*-deficient strain, it is unlikely that the host bacteria's DNA replication machinery is completely inhibited. This suggests that *E. coli* has an alternative DNA replication mechanism that compensates when the *priA* gene is deleted. The activation of this PriA-

independent replication pathway may explain the increased phage susceptibility observed in the *priA*-deficient strain. Damage to the DNA or stress-induced SOS responses necessitates that the replication fork be repaired and restarted. *E. coli* exhibits three restart pathways: PriA/PriB, PriA/PriC, and PriC/Rep (McKenzie et al., 2022). While PriA is vital for restarting replication, the PriC pathway can also restart replication in the absence of PriA (Leroux et al., 2017). This suggests that *E. coli* has an alternative DNA replication system even without the *priA* gene, which could explain the heightened phage sensitivity in *priA*-deleted mutants.

Certain compounds can suppress PriA function by either disrupting its interaction with the single-strand binding protein (SSB) or by inhibiting its ATPase activity (Huang et al., 2015; Voter et al., 2018). For instance, kanamycin and chloramphenicol are known to interfere with the PriA-SSB interaction (Voter et al., 2018). Research has indicated that chloramphenicol concentrations below 40 μM are effective in inhibiting PriA function. These antibiotics inhibit protein synthesis by binding to the 30S and 50S ribosomal subunits of bacteria, respectively (Bulkley et al., 2010; Chulluncuy et al., 2016). To minimize bactericidal effects while inhibiting PriA function, this study used low concentrations of chloramphenicol (2 and 5 μmol/L). Additionally, naturally derived substances without antibiotic properties were also investigated for their ability to inhibit PriA. One such substance, kaempferol, a flavonoid, was found to inhibit PriA by blocking its ATPase activity (Huang et al., 2015). In this study, kaempferol showed no antibacterial activity at the concentrations used, with a MIC of more than 512 μg/mL (1.79 mmol/L) (Zhou et al., 2022). However, even at a high concentration of 1 mmol/L, kaempferol did not significantly enhance the phage's effect on *E. coli*, likely due to the external layer of gram-negative bacteria, which makes it challenging for flavonoids to penetrate the cell. Conversely, the effects were more pronounced when kaempferol was used with phages on gram-positive bacteria such as *Enterococcus faecalis* (data not shown). Overall, the research observed that kanamycin and chloramphenicol, which

interfere with PriA functionality, increased phage vulnerability in *E. coli* BW25113 and delayed the recovery of phage-resistant cells. The study investigated the impact of combining phages with antibiotics, specifically chloramphenicol or kanamycin, on *E. coli* O157:H7. The results indicated that this combined treatment could significantly enhance the efficacy of phage therapy. This finding suggests a promising approach for improving the effectiveness of phage therapy in combating bacterial infections. In our initial study, deleting nine additional genes seemed to enhance phage susceptibility for several reasons. For instance, the *yfcD* gene is thought to encode a Nudix hydrolase (NUDX), which breaks down nucleoside diphosphate analogs (Morrison et al., 2016). NUDX is involved in metabolic regulation, stress-induced cell death, and defense mechanisms (Yoshimura and Shigeoka, 2015). Therefore, the absence of *yfcD* likely reduces NUDX activity, increasing vulnerability to phages. The *rpe* gene encodes D-ribulose-5-phosphate 3-epimerase, an enzyme critical for the pentose phosphate pathway that generates reducing agents like NADPH (Tan et al., 2016). Deleting *rpe* disrupts this pathway, resulting in poor *E. coli* growth (Lyngstadaas et al., 1998), which may increase phage susceptibility due to impaired cellular functions. Additionally, the *gor* gene encodes glutathione oxidoreductase, an enzyme essential for converting oxidized glutathione to its reduced form, which is crucial for maintaining cellular antioxidant defenses (Greer and Perham, 1986). The loss of *gor* impairs this defense mechanism, making the bacteria more prone to phage attacks. In summary, the deletions of these genes affect various cellular pathways and defense mechanisms, leading to increased sensitivity of *E. coli* to phages. The *ubiE* gene encodes an enzyme involved in ubiquinone biosynthesis, encoding a methyltransferase. Ubiquinone functions as a cofactor in the electron-transfer system and as an antioxidant (ArcA-ArcB system) (Lee et al., 1997). Deletion of *ubiE* disrupts these functions, potentially increasing phage susceptibility. The function of *yaiW* remains unclear, but it is known to be a cell surface lipoprotein involved in environmental signal transduction. Loss of *yaiW* may affect

phage infectivity due to changes in cell surface structure and membrane potential maintenance. The function of the *ybcH* gene product in *E. coli* is not currently known. The genes *tolR* and *tolA* are part of the Tol-Pal system, essential for membrane stability in gram-negative bacteria (Wojdyla et al., 2015). Deletion of these genes destabilizes the cell membrane, enhancing phage vulnerability. The *dnaK* gene codes for the heat shock protein DnaK, a molecular chaperone involved in protein folding and refolding (Mayer and Bukau, 2005). Loss of *dnaK* likely leads to protein aggregation, including proteins involved in phage defense, increasing phage susceptibility, further investigation is required to identify which protein refolding is critical in phage defense. Several phage defense mechanisms have been reported in bacteria, such as preventing phage adsorption through receptor deficiency, the CRISPR/Cas system, extracellular matrix production, physical inhibition of phage binding, and programmed cell death to protect other cells. The CRISPR/Cas system, for example, excises and remembers parts of foreign genes, such as phage genomes, to defend against subsequent infections. The *cmr* gene is involved in this system, and its deletion leads to decreased turbidity at 6 h post-infection but increased turbidity at 48 h, indicating incomplete inhibition of phage resistance with a single gene deletion. Other defense mechanisms encompass a variety of strategies, such as blocking the entry of phage DNA into the cell, cleaving phage genomes via the restriction-modification system, initiating programmed cell death, and producing an extracellular matrix. The extracellular matrix, composed of a complex network of polysaccharides and proteins, serves as a physical barrier, preventing phage particles from accessing the bacterial cell surface. Additionally, by inhibiting the entry of phage DNA, bacteria can thwart the infection process at an early stage, thereby enhancing their defense against phage attacks. Programmed cell death further prevents the spread of phages by sacrificing infected cells. These mechanisms can be either natural or acquired in response to phage infection. The experimental system used 96-well plates for screening due to the large number of test strains, which may have

affected the frequency of contact between bacteria and phages compared to test tube experiments. The primary screening method using toothpicks for seeding bacteria may have introduced variability in MOI between samples. Despite repeated secondary screening and antimicrobial testing, some defective strains not selected in the primary screening may have been important for phage resistance.

In this study, cells treated with phage for 16 h exhibited a significant increase in the transcription level of *priA* compared to cells that were not exposed to phage. This suggests that phage treatment triggers an upregulation of *priA*, highlighting its potential role in the bacterial response to phage infection. At this point, most cells had become phage-resistant cells. However, phage resistance decreased in the presence of a substance at low concentrations (5 μmol/L) (Fig. 2.10). If PriA is crucial for phage resistance and this resistance is not due to mutations in the receptor, it might be possible to kill the phage-resistant cells again using the same phage combined with PriA inhibitors at concentrations sufficient to inhibit PriA function. Additionally, the 10 mutants with deleted genes selected in this study do not cover all the genes involved in the phage resistance mechanism. Further research on genes involved in the CRISPR/Cas system and creating strains deficient in multiple genes or inhibiting these genes' functions together will help clarify phage resistance mechanisms. This study represents the first investigation into screening for phage resistance genes using the Keio collection, while also exploring the role of PriA in both phage resistance and susceptibility. The results highlight the importance of these genes in phage defense and suggest potential targets for enhancing phage therapy.

2.5. Summary

Chapter 2 revealed that 10 mutants with deleted genes were selected for phage resistance by screening the 3,909 single-gene mutants of *E. coli* BW25113 from the Keio collection. The *priA* mutant showed the most significant increased vulnerability to the phages S127 BCL3 and T7. Additionally, *E. coli* BW25113 exhibited increased phage susceptibility when exposed to chloramphenicol or kanamycin, which inhibit PriA at sub-MIC concentrations. The regrowth of phage-resistant *E. coli* BW25113 cells was suppressed when combined with phage and these substances. Similar effects were observed in *E. coli* O157:H7, suggesting a promising combined effect for phage therapy. Phage defense strategies in bacteria are varied, and it is typical for bacteria to have multiple mechanisms to counteract phage attacks. Given this variety, bacteria are believed to combat phage threats by employing a combination of different defense tactics. In light of this, investigating further genes that play a role in phage defense, along with the functions of PriA, and exploring how they operate together in foodborne pathogens could aid in preventing the emergence of phage-resistant pathogens.

Table 2.1. Primers designed and used in quantitative real-time PCR.

Gene	[a]Primer sequence 5'-3'	Primer length
priA	F: GCGATTGAATACCGAACAGG	20
	R: CCAGTACGCTGAGATAAACC	20
16S rRNA	F: GTGAAATGTTGGGTTAAGTC	20
	R: AGTTTATCACTGGCAGTCTC	20

[a]F: forward, R: reverse.

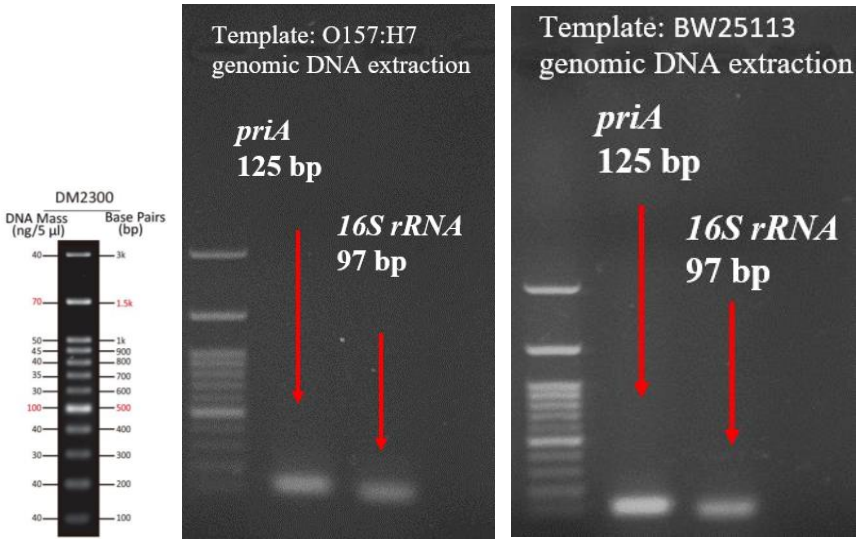


Fig. 2.2. The specificity of the primers.

Table 2.2. The lytic spectra of isolated phage S127BCL3

Tested strains	Source of strains	Lytic activity
Salmonella Typhimurium NBRC12529	Biological Resource Center, Tokyo Japan	-
Staphylococcus aureus NCTC8325	UK Health Security Agency, Salisbury, UK	-
Listeria monocytogenes ATCC15313	American Type Culture Collection, USA	-
Bacillus cereus JCM2152	RIKEN BioResource Research Center, Ibaraki, Japan.	-
Escherichia coli O157:H7 (EC-071)	Fukuoka City Institute of Health and Environment, Fukuoka, Japan	+
Escherichia coli BW25113	National Institute of Genetics, Shizuoka, Japan	+

+: positive

-: negative

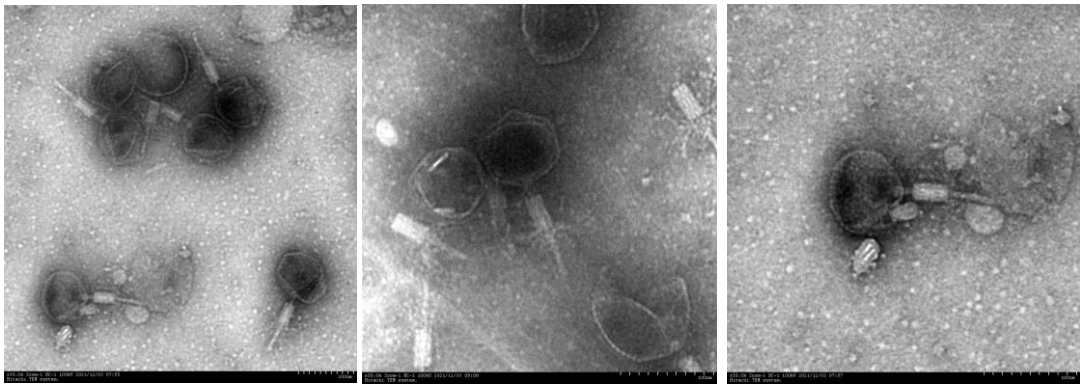


Fig. 2.3. Transmission electron microscopy image of phage S127BCL3. The head and tails were visible. The existence of a contractile tail indicates that this phage is a member of the *Myoviridae* family. Scale: 100 nm.

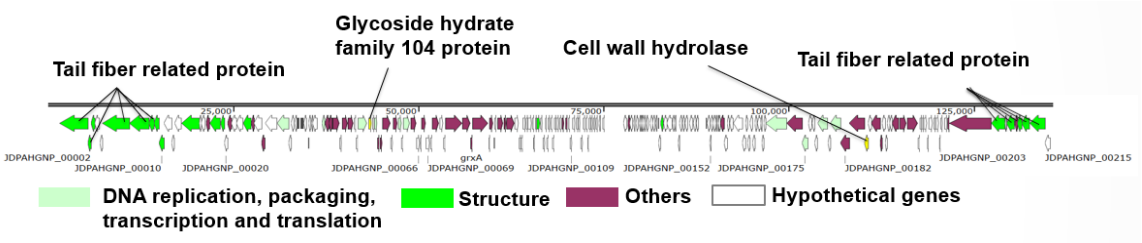


Fig. 2.4. Genetic layout of phage S127BCL3. The double-helical DNA genome of phage S127BCL3 contains genes for two lytic enzymes (cell wall hydrolase and glycoside hydrate). With a genome length of 135,530 bp, it is presumed that ten of these genes code for tail fiber proteins. Based on genome similarity, phage S127 BCL3 could be reclassified into the *Vequintavirinae* family, similar to phage vB_EcoM-ECP26.

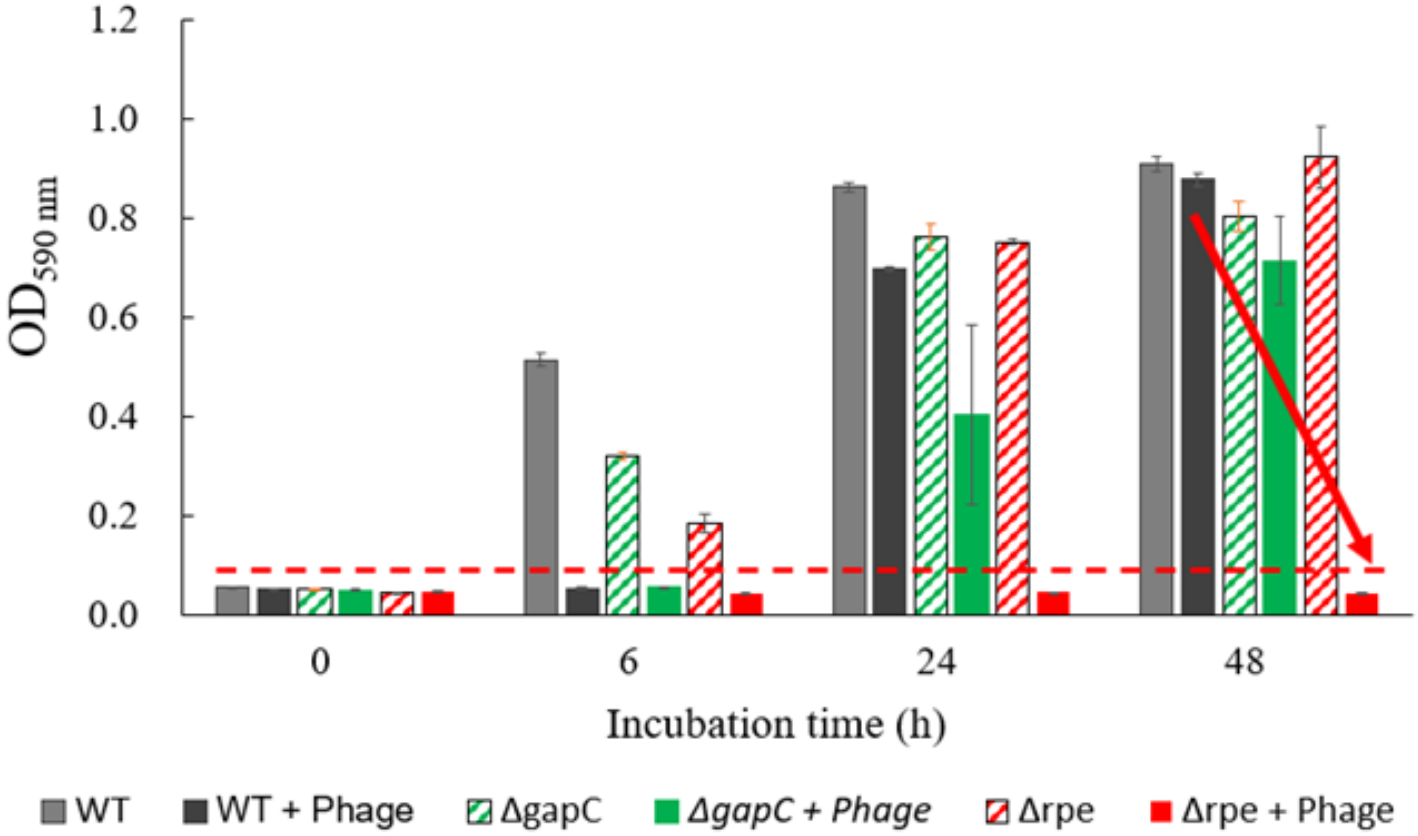


Fig. 2.5. Effect of phage S127BCL3 on *E. coli* BW25113, *ΔgapC* and *Δrpe* mutants. WT (Wild-type) refers to the parental *E. coli* BW25113 strain without any gene deletions. The *E. coli Δrpe* gene-deletion mutant strain (red color) represents nine other strains (*ΔyfcD;*JW2296, *ΔybcH;*JW0556, *ΔyaiW;*JW0369, *Δgor;*JW3467, *ΔubiE;*JW5581, *ΔdnaK;*0013, *ΔtolR;*JW0728, *ΔpriA;*JW3906, and *ΔtolA;*JW0729) that did not show increased turbidity 24 and 48 h after phage treatment. Conversely, the *E. coli ΔgapC* gene-deletion mutant strain (green color) represents those that did exhibit increased turbidity at these times.

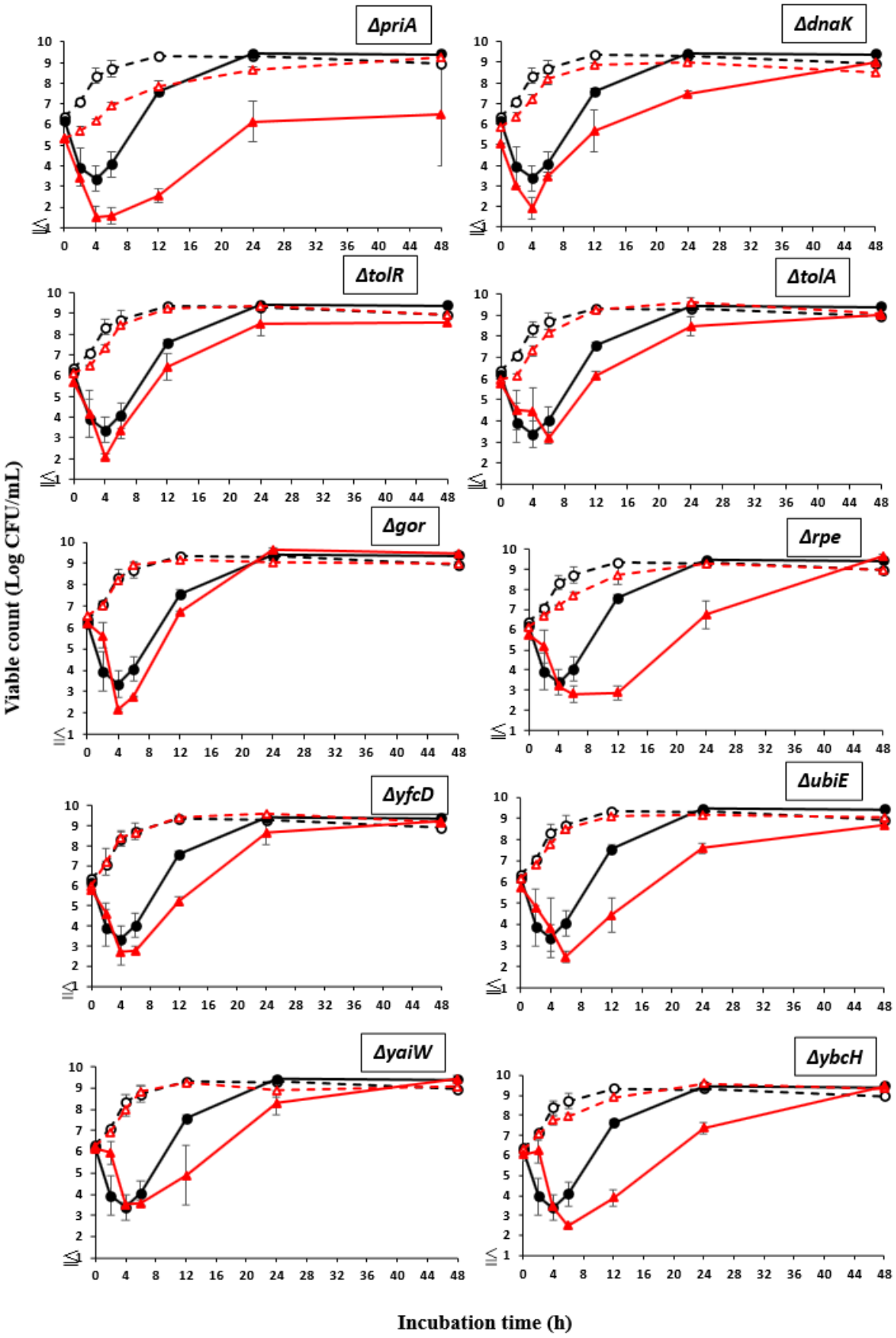
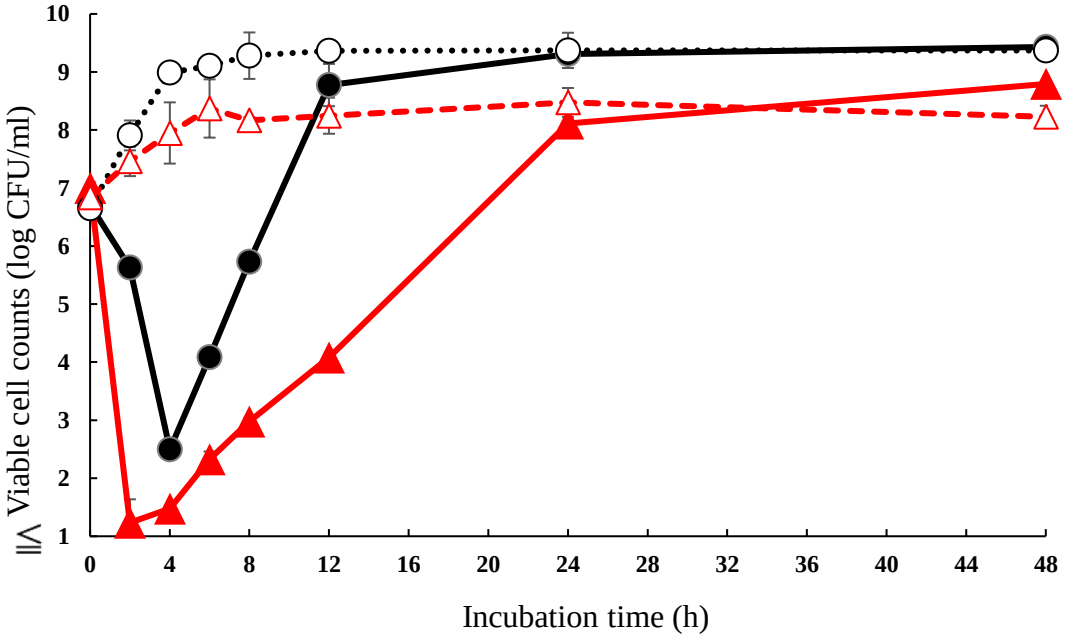


Fig. 2.6. Impact of phage S127 BCL3 on *E. coli* BW 25113 (○, ●) and ten single-gene deletion mutants (△, ▲), incubated with (filled symbols, solid lines) and without (open symbols, dashed lines) the phage at an MOI of 10. Values are means ± SD for 3 separate experiments.

(a) phage S127BCL3



(b) phage T7

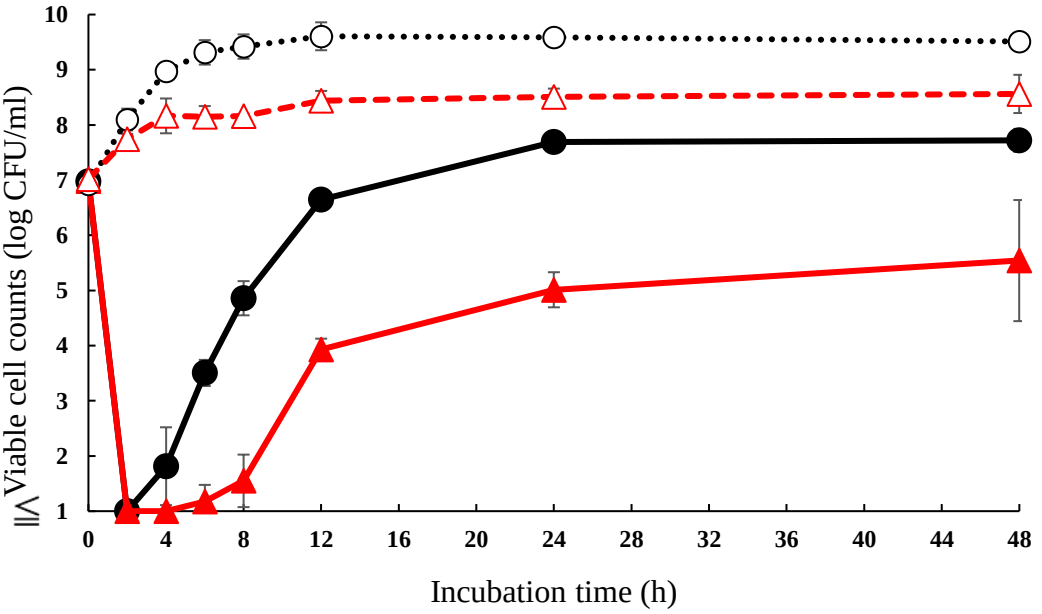


Fig. 2.7. Comparative analysis of susceptibility between *E. coli* BW25113 (○, ●) and *ΔpriA* mutant (△, ▲) to phage S127BCL3 (a) and T7 (b). The experiments involved incubation with phage S127BCL3 at an MOI of 0.01, indicated by filled symbols, and without phage, indicated by open symbols. The data represent the mean values ± standard deviation from three independent experimental replicates.

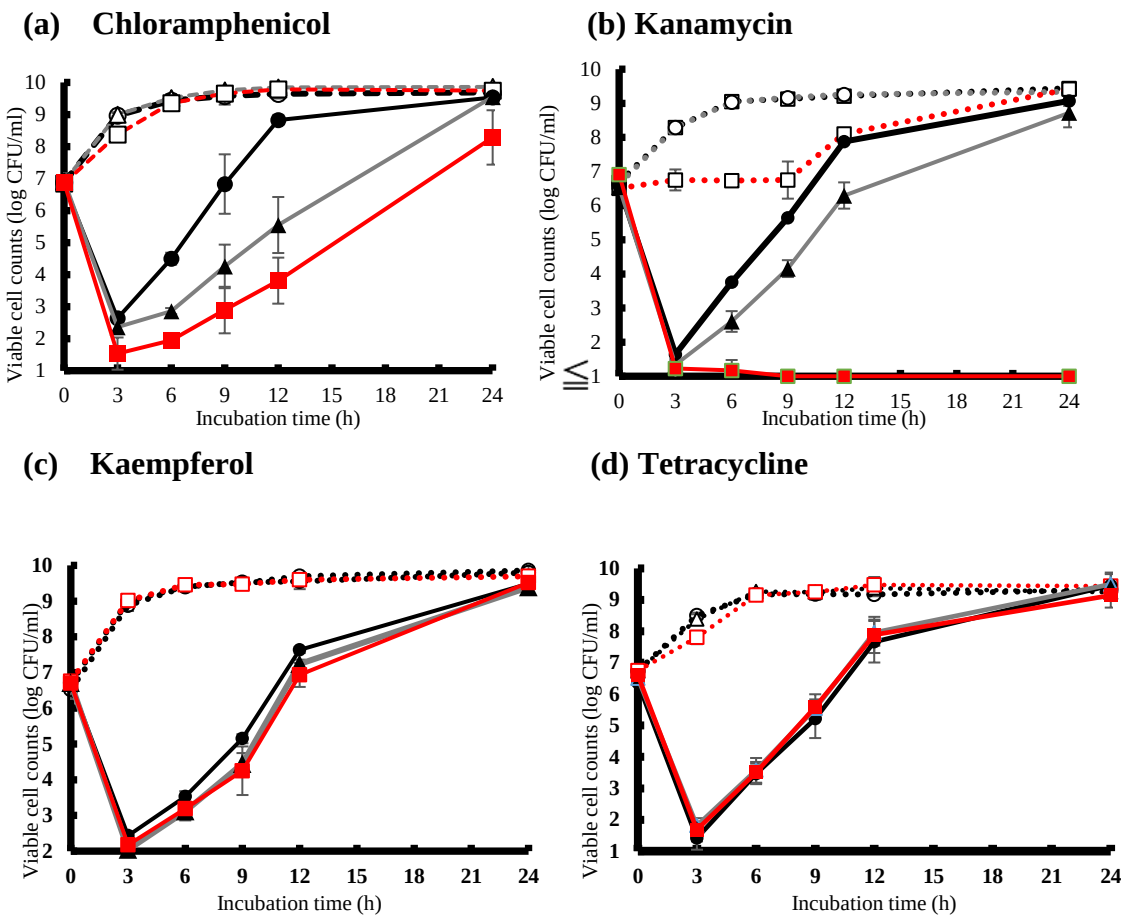
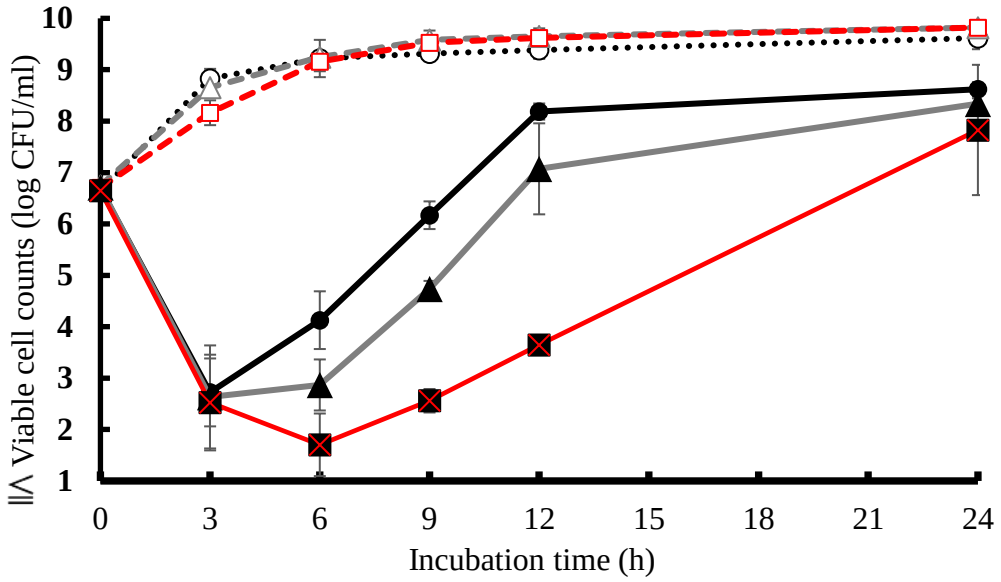


Fig. 2.8. Impact of chloramphenicol (a), kanamycin (b), kaempferol (c), and tetracycline (d) on the phage vulnerability of *E. coli* BW25113. The experiments included cultures of *E. coli* with (filled symbols) and without (open symbols) phage S127BCL3 at an MOI of 0.001. Tetracycline was tested at concentrations of 0 (○, ●), 0.5 (△, ▲), and 1 (□, ■) µmol/L; kanamycin at 0 (○, ●), 2 (△, ▲), and 5 (□, ■) µmol/L; chloramphenicol at 0 (○, ●), 2 (△, ▲), and 5 (□, ■) µmol/L; and kaempferol at 0 (○, ●), 0.5 (△, ▲), and 1 (□, ■) mmol/L. These data represent the mean values ± standard deviation from three independent experiments.

(a) Chloramphenicol



(b) Kanamycin

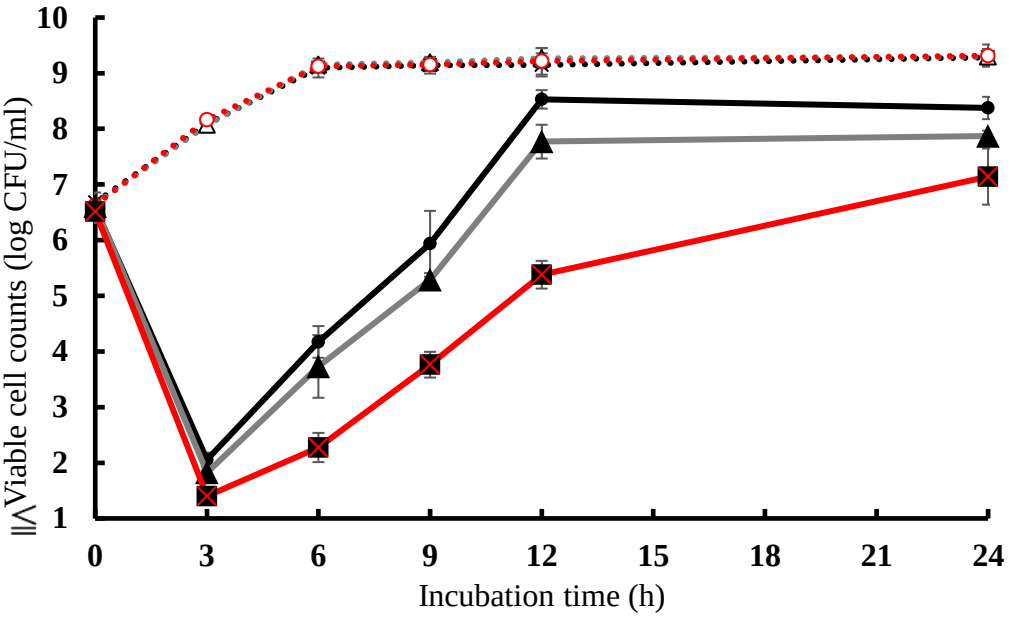


Fig. 2.9. Effect of chloramphenicol (a) and kanamycin (b) on the phage vulnerability of *E. coli* O157:H7. The bacteria were cultured with (filled symbols) and without (open symbols) phage S127BCL3 at an MOI of 0.01. Chloramphenicol concentrations tested were 0 (○, ●), 2 (△, ▲), and 5 (□, ■) μmol/L, while kanamycin concentrations were also tested at 0 (○, ●), 2 (△, ▲), and 5 (□, ■) μmol/L. The values represent means ± standard deviation from three separate experiments.

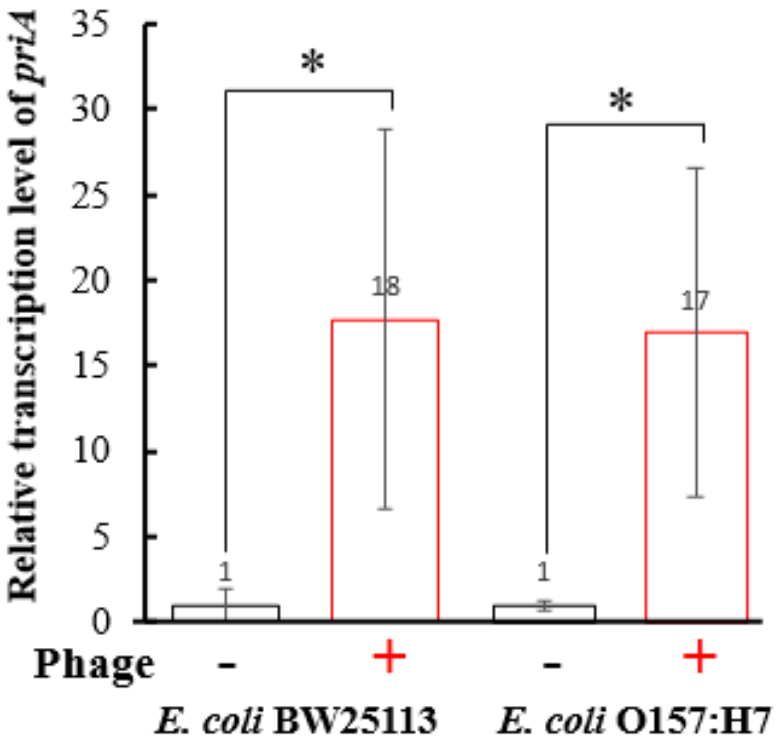


Fig. 2.10. Comparison of *priA* gene expression in phage-resistant and -sensitive *E. coli* BW25113 and O157:H7 populations. The experiment involved incubating the bacteria with phage S127BCL3 at an MOI of 0.001 for 16 h, alongside a control without phage. After incubation, total RNA was extracted, and qPCR was used to quantify *priA* transcription levels. The data are presented as means ± standard deviation from three independent experiments, with statistical significance denoted by asterisks (*) indicating $p < 0.05$

Chapter 3. Control of phage-resistant *Escherichia coli* populations through PriA inhibition and flavonoid application

3.1. Introduction

The development of bacterial populations that are resistant to phages poses a significant challenge to the use of bacteriophages in food applications. To address this burden, chapter 2 has confirmed the *priA* gene involving in *E. coli*'s resistance to phages and the function of PriA inhibition contributed to slow the resurgence of *E. coli* populations resistant to phages. Additionally, the transcription level of *priA* was found to rise significantly in populations resistant to phages. If the expression of PriA was essential for resistance to phage S127 BCL3 and the phage receptors in the resistant cells remain unchanged, it is feasible that these phage-resistant cells could be susceptible to reinfection by the same phage combined with PriA inhibitors at doses sufficient to suppress the enhanced function of PriA. Therefore, it is necessary to examine the phage-resistant populations.

However, phage resistance is governed by multiple genes through various mechanisms. In our preliminary experiment, among the 3,909 strains, nine other deletion mutant strains (*ΔdnaK;JW0013, Δgor;JW3467, ΔtolA;JW0729, ΔtolR;JW0728, ΔubiE;JW5581, Δrpe;JW3349, ΔybcH;JW0556, ΔyaiW;JW0369,* and *ΔyfcD;JW2296*) also exhibited a higher phage vulnerability compared to the parental strain of *E. coli* BW25113 (Fig. 2.5). Therefore, the synergistic impacts of PriA inhibition and targeting other resistance mechanisms associated with different genes could lead to greater suppression of regrowth in phage-resistant populations. There is study reported that the chaperone protein DnaK involving in CRISPR adaptation through the interaction with the Cas1–Cas2 complex and modulating its activity, specifically by inhibiting the DNA binding and integration abilities of the complex, which are essential for the capture and integration of mobile genetic elements (MGEs) DNA into CRISPR sites in *Escherichia coli* (Killelea et al., 2023). They found that DnaK inhibits the naive adaptation process

in cells without pre-existing immunity. This process is critical for the capture and integration of MGE DNA by Cas1–Cas2. The inhibition by DnaK is a protective mechanism to prevent erroneous targeting of the host's own DNA. However, the study suggests that under certain conditions, such as the lack of DnaK or the existence of phage proteins, this inhibition can be lifted, allowing the CRISPR system to acquire new spacers from invading MGEs effectively. It highlights the role of DnaK in regulating the CRISPR system's ability to acquire new genetic material, ensuring the precise targeting of foreign DNA while preventing self-targeting. This connection suggests a sophisticated level of regulation and targeting in the CRISPR-Cas system, facilitated by the interaction with DnaK.

The versatile DnaK chaperone system, comprising the DnaK, DnaJ, and GrpE complex, plays a fundamental role in *Escherichia coli* by ensuring the proper folding, repair, and degradation of proteins. This system is indispensable for maintaining essential cellular functions across a range of conditions, including both normal physiological states and various stress conditions. The significance of the DnaK chaperone system extends to its involvement in critical processes such as protein homeostasis, stress response, and recovery from protein denaturation (Mayer and Bukau, 2005). The principal heat shock proteins in bacteria include DnaK, DnaJ, GroEL, and HtpG. These proteins are vital for bacterial survival and adaptation, especially under thermal and other environmental stresses. Notably, the DnaK and DnaJ proteins work together to facilitate the correct folding of newly synthesized and stress-damaged proteins. This pair is crucial in preventing the aggregation of misfolded proteins, thereby ensuring cellular viability and function during stressful conditions. The joint action of DnaK and DnaJ supports protein folding and plays a critical role in the bacterial stress response, helping the cell to withstand and recover from adverse situations.(Genevaux et al., 2007). DnaK, commonly referred to as heat shock protein Hsp70, has a molecular mass of 70 kDa and is predominantly localized in the cytoplasm. This protein is essential for maintaining cellular homeostasis by preventing protein

misfolding, especially during cellular stress responses (Hesterkamp and Bukau, 1998). In bacteria, DnaK is indispensable for survival when exposed to environmental stressors such as high temperatures, increased levels of heavy metals, or the presence of antibiotics (Mayer et al., 2000). The ATPase activity of DnaK, which is stimulated by DnaJ, can be suppressed by preventing the assembly of the DnaK-DnaJ interaction in vitro (Cesa et al., 2013; Chang et al., 2011). Myricetin has been reported to inhibit the cellular function of DnaK (Chang et al., 2011). This widely recognized plant flavonoid, present in many food ingredients, holds potential nutraceutical benefits. (Sato et al., 2013). Myricetin (Fig. 3.1) is noted for its pharmacological properties, which include antioxidant, cytoprotective, anticancer, antiviral, antiplatelet, anti-inflammatory, and lipid-lowering effects Ong and Khoo, 1997). Additionally, Numerous studies have demonstrated that flavonoids possess antimicrobial activity against pathogens. (Júnior et al., 2018). In this chapter 3, the *dnaK* gene knockout mutant strain of *E. coli* BW25113 was selected for the investigation of the synergistic impact with PriA inhibition. Subsequently, the combined effects of myricetin with PriA inhibitor (chloramphenicol) on the phage vulnerability of *E. coli* BW25113 and O157:H7 were investigated.

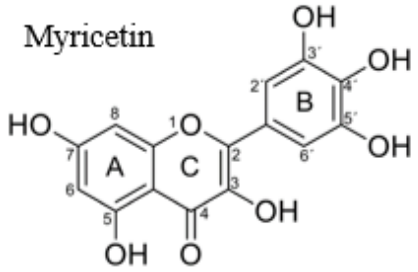


Fig. 3.1. The flavonoids used in this research have a specific molecular structure.

3.2. Materials and methods

3.2.1. Bacterial strains, inoculum preparation, and culture conditions

The *E. coli* O157:H7 strain (EC-071) was supplied by the Fukuoka City Institute of Health and Environment in Fukuoka, Japan. Meanwhile, *E. coli* BW25113, the parental strain that lacks kanamycin resistance, along with kanamycin-resistant single-gene knockout strains, were acquired from the National Institute of Genetics in Shizuoka, Japan. The bacterial strains were preserved in a microbank at −80 °C. The single-gene knockout strains were cultured in LB and TSA media supplemented with 200 mg/L kanamycin. Before use, the bacterial strains were streaked onto tryptic soy agar (TSA; Becton Dickinson and Company, Sparks, MD, USA) to obtain isolated colonies. A single colony was then selected and transferred into 5 mL of Luria Bertani broth (LB; Becton Dickinson and Company, Sparks, MD, USA). The inoculated broth culture was incubated overnight at 37 °C with continuous shaking at 130 rpm. This incubation period allowed the cells to reach the stationary growth phase, ensuring optimal conditions for subsequent experimental procedures. The culture's optical density (OD) was assessed using a UV-160 spectrophotometer (Shimadzu, Japan) at 600 nm to determine cell concentrations. Following measurement of the optical density, the culture was diluted with LB broth to achieve approximately 10^8 CFU/mL (OD_{600} = 0.1 for BW25113 and O157:H7 strains, 0.3 for the *ΔdnaK* mutant strain, and 0.6 for the *ΔpriA* mutant strain). This bacterial suspension was then employed in subsequent experiments.

3.2.2. Propagation of the phage S127BCL3

Phage S127BCL3 was used from the chapter 2 isolated from chicken liver. For propagation, this phage S127BLC3 was mixed with *E. coli* BW25113 or O157:H7 (EC-071, its original host strain) and incubated overnight at 37°C in LB medium with shaking for exponential growth. Phage S127BLC3, propagated with *E. coli* BW25113, was utilized to infect both *E. coli* BW25113 and the single-gene knockout mutants. Additionally, Phage S127BLC3, propagated with *E. coli* O157:H7, was used to infect *E. coli* O157:H7. Post-culture, the mixture was centrifuged at 12,000 × *g* for 5 minutes at 4°C. The phage solution was obtained by filtering the supernatant through a 0.22 µm pore size sterilization filter (As One Corp., Osaka, Japan) and then stored at 4 °C. The

phage stock had a titer of 10^{10} PFU/mL, calculated from the number of plaques formed after overnight incubating process. The phage was then diluted in saline magnesium (SM) buffer, consisting of 0.05 mol/L Tris-HCl buffer (pH 7.5) with 0.1 mol/L NaCl, 0.008 mol/L $MgSO_4$, and 0.01% gelatin.

3.2.3. Preparation of myricetin and chloramphenicol

Myricetin, sourced from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) with a purity exceeding 97.0%, was mixed with 100% dimethyl sulfoxide (DMSO, Nacalai Tesque Co., Ltd., Japan) at a concentration of 50 mmol/L. The solutions underwent sterilization via filtration using a Millex®-FG membrane filter (0.20 μm pore size, Millipore, Carrigtwohill, Co., Inc., Cork, Ireland). Chloramphenicol from Nacalai Tesque, Inc., Japan, was prepared at a concentration of 20 mg/mL in 99.5% ethanol, filtered, and diluted to 5 mmol/L. All solutions were stocked at −4 °C before the experimental use.

3.2.4. Selection of phage-resistant *E. coli* O157:H7 strains

E. coli O157:H7 strains (EC-071) were grown overnight at 37 °C in 5 mL of Luria Bertani (LB) medium with agitation at 130 rpm. 500 μL of bacterial suspension (containing 10^8 CFU/mL) was introduced to a test tube containing 4 mL of LB containing 1 mmol/L $CaCl_2$. Then, 500 μL of phage solution, standardized to a concentration of 10^5 PFU/mL with a multiplicity of infection (MOI) of 0.001, was introduced and mixed with the bacterial suspension, then cultured at 37 °C with agitation at 130 rpm for 16 h to obtain phage resistant cells in the stationary phase of growth. The resistant bacterial resistant strains were streaked onto tryptic soy agar (TSA; Becton Dickinson and Company, Sparks, MD, USA) and incubated at 37 °C overnight until the colonies formed. A single colony was randomly picked and repeated the phage treatment process again for twice infection with phage. After the second infection, the single colonies were randomly picked on the TSA and confirmed the phage resistance by spot test method with the phage solution, standardized to a concentration of 10^{10}

PFU/mL. The phage-resistant strains were stocked at -80 °C in 15% glycerol for the experimental use.

3.2.5. Phage reinfection treatment of phage-resistant *E. coli* O157:H7 strains

To explore the impacts of enhanced PriA inhibition on phage vulnerability of phage-resistant *E. coli* O157:H7, the following method was conducted. In a test tube with 4.5 mL of LB broth containing 1 mmol/L $CaCl_2$, 0.5 mL of a phage-resistant *E. coli* O157:H7 suspension (10^3 CFU/mL) was added to reach a final concentration of 10^2 CFU/mL. For the treatment group, 5 μL of chloramphenicol solution was added to achieve 5 μmol/L. Both groups were incubated at 37 ℃ with constant shaking for 6 h. Controls received 5 μL of 99.5% ethanol instead. After 6 h, 50 μL of phage S127BCL3 was added to achieve final concentrations of 10^8 PFU/mL (without chloramphenicol) and 10^6 PFU/mL (with chloramphenicol), ensuring an MOI of 10. Control groups received SM buffer instead of the phage. The mixtures were incubated at 37 ℃ with shaking at 130 rpm for 24 h.

3.2.6. Myricetin and phage treatment of *E. coli ΔpriA* mutant strain

To explore the effects of myricetin on the phage vulnerability of *ΔpriA* mutant strain, the following method was employed. A total of 4.5 mL of LB broth containing 1 mmol/L $CaCl_2$ was prepared in a test tube. Then, 0.5 mL of *E. coli ΔpriA* mutant cell suspension at concentrations of 10^8 CFU/mL and 10^6 CFU/mL was introduced to achieve final concentrations of 10^7 CFU/mL (for the flavonoid treatment) and 10^5 CFU/mL (for the control without flavonoids), respectively. Next, 50 μL of flavonoid solutions were added to the cell suspensions to reach a final concentration of 500 μmol/L for myricetin treatment. The cell suspensions were shaken constantly and incubated at 37°C for 6 h. For the control samples, 50 μL of DMSO was used instead of the flavonoid solution. After the 6-hour incubation, 50 μL of phage S127BCL3 suspension was introduced to achieve final concentrations of 10^8 PFU/mL (MOI=10) and 10^4 PFU/mL (MOI=0.001), respectively, and incubated at 37°C. In the control

samples, instead of adding the phage suspension, an equivalent volume of SM buffer was used in the controls. The mixtures were then incubated at 37°C with agitation at 130 rpm for 48 h.

3.2.7. Chloramphenicol and phage treatment of *E. coli ΔdnaK* mutant strain

To explore the effects of chloramphenicol on the phage vulnerability of the *ΔdnaK* mutant strain, the following procedure was utilized. A test tube containing 4.5 mL of LB broth containing 1 mmol/L $CaCl_2$ was prepared. To this broth, 0.5 mL of an *E. coli ΔdnaK* mutant cell suspension with a concentration of 10^7 CFU/mL was added to achieve a final concentration of 10^6 CFU/mL. The cell suspensions were shaken continuously and incubated at 37°C for 6 h. After this incubation period, 5 µL of chloramphenicol solution was added to achieve a final concentration of 5 µmol/L for the chloramphenicol treatment. For the control group, 5 µL of 99.5% ethanol was used instead of the chloramphenicol solution. Subsequently, 0.5 mL and 50 µL of phage S127BCL3 suspension were introduced to reach final concentrations of 10^9 PFU/mL (MOI=10) and 10^6 PFU/mL (MOI=0.01), respectively, and incubated at 37°C. In the control samples, instead of the phage suspension, an equal amount of SM buffer was substituted to the cell suspensions, then incubated at 37°C with constant shaking for 6 h. The mixtures were incubated at 37°C with agitation at 130 rpm for 48 h.

3.2.8. Combined treatment with chloramphenicol and phage of *E. coli* BW25113 and O157:H7 pretreated with myricetin

To explore the combined impacts of flavonoids and chloramphenicol on the phage vulnerability of *E. coli* BW 25113, the following method was utilized. In a test tube containing 4.5 mL of LB broth containing 1 mmol/L $CaCl_2$, 0.5 mL of cell suspension with concentrations of 10^7 CFU/mL and 10^6 CFU/mL was introduced to achieve final concentrations of 10^6 CFU/mL (with/without flavonoid treatment) and 10^5 CFU/mL (control without flavonoids), respectively. To these cell suspensions, 50 µL of

myricetin solutions were introduced to reach a final concentration of 500 μmol/L. Afterward, the suspensions were subjected to constant shaking at 37°C for 6 h. In the control samples, the myricetin solution was substituted with 50 μL of DMSO. After the 6-hour incubation, 0.5 mL of phage S127BCL3 suspension was introduced to achieve a final concentration of 10^9 PFU/mL (MOI=1) and incubated at 37°C. In the control samples, an equal volume of SM buffer was introduced instead of the phage suspension. The mixtures were incubated at 37°C with agitation at 130 rpm for 48 h. Similar experiments using myricetin were conducted on *E. coli* O157:H7 (EC-071).

3.2.9. Viable count determination

Samples of 20 μL were taken from each tube at 0, 6, 9, 12, 24, and 48 h. These samples were then serially diluted 10-fold with phosphate buffered saline (PBS, pH 7.4, containing 137 mmol/L NaCl, 8.10 mmol/L Na_2HPO_4, 2.68 mmol/L KCl, and 1.47 mmol/L KH_2PO_4). For *E. coli* O157:H7, the pH of PBS was adjusted to 2 (acid condition) for the purpose of reducing the phage-produced endolysin influences on the spotted samples. Then, 10-μL and 100-μL aliquots of each dilution were spotted or spread on TSA plates. Following overnight incubating process at 37°C, viable cell counts were determined by counting colonies.

3.2.10. Statistical evaluation

The experiments were conducted three times for each condition to ensure reliability and reproducibility of the results. The data are presented as mean values accompanied by standard deviations to reflect variability. For statistical analysis, a one-way analysis of variance (ANOVA) followed by a *t*-Test was conducted using Microsoft Excel 2016 for Windows to determine significant differences between the treatment and control groups. A *p*-value of less than 0.05 was considered indicative of statistical significance. This rigorous approach ensured that the findings were robust and that any observed differences were unlikely to be due to random chance.

3.3. Results

3.3.1. Effects of PriA inhibitor on phage vulnerability of phage-resistant *E. coli* O157:H7

In chapter 2, the transcription level of *priA* increased significantly among phage-resistant *E. coli* populations (Figure 2.10). To examine if these phage-resistant cells could be susceptible to reinfection by the same phage combined with PriA inhibitors, the selection strains of phage-resistant *E. coli* O157:H7 were pretreated with 5 μmol/L of chloramphenicol for 6 h before the same phage S127 BCL3 addition. As shown in Fig. 3.2, in the lack of chloramphenicol, there was no significant difference observed in the viable counts between the control (without phage treatment) and the phage-resistant cells cultured with phage alone. However, the viable cell counts in the existence of 5 μmol/L chloramphenicol for 6 h were 2 log lower than those of cells without chloramphenicol pretreatment. The phage-resistant cells showed antibiotics sensitivity again after 6 h at 5 μmol/L of chloramphenicol without phage treatment. After 3 h and 6 h of phage addition in the existence of chloramphenicol, the viable counts of the resistant cells were respectively 4.8 log and 5.9 log CFU/mL, which were respectively 1.3 and 1.6 log lower than those of the cells without the phage addition. Therefore, phage sensitivity significantly increased among phage-resistant cells at 9 and 12 h of chloramphenicol incubating process. It is interesting to observe that the viable counts of the resistant cells remain unchanged after 3 h of phage treatment with chloramphenicol, maintaining the same level as when in a resting state.

3.3.2. Impact of myricetin treatment duration on phage vulnerability of *E. coli*

To explore the impacts of myricetin treatment duration on the phage vulnerability of *E. coli* BW25113 (wild-type strain), *E. coli* cultures were initially exposed to a pretreatment process involving 500 μmol/L of myricetin from 0 to 12 h before phage exposure (Fig. 3.3). The viable cell counts were similar with and without myricetin when the phage was added after 0 and 3 h of incubating process. However, during the

pretreatment with myricetin at 6 and 9 h, there was a significant increase in phage sensitivity. This enhanced susceptibility was observed consistently, indicating the effective impact of myricetin on the bacterial cells over these time periods (Fig. 3.3c and 3.3d). *E. coli* BW25113 cells that were pretreated with myricetin for 6 and 9 h exhibited viable counts that were 1.5 and 1.3 logs lower, respectively, compared to untreated cells after 6 h of phage exposure. This indicates a substantial reduction in bacterial viability due to the combined effects of myricetin pretreatment and phage exposure. Consequently, a 6-hour myricetin pretreatment was chosen for subsequent experiments.

3.3.3. Impacts of myricetin and phage on the control of viability and regrowth of *E. coli ΔpriA* mutant strain

To investigate the effects of myricetin on phage vulnerability of *ΔpriA* mutant strain, the strain underwent pretreatment with 500 µmol/L myricetin for 6 h and then added with phage S127BCL3 after the pretreatment. The MOIs were set to be high and low with the phage S127BCL3 final concentration of 10^8 PFU/mL (MOI=10) and 10^4 PFU/mL (MOI=0.001), respectively. Regardless of the MOI being high or low, the viable count in the myricetin treatment group was 1 log lower than those of cells that did not receive myricetin after 6 h of phage exposure (Fig. 3.4). However, after 24 h of incubating process, the viable count in the myricetin treatment group with phage at high MOI was 2 logs lower compared to the group with phage alone (Fig. 3.4a), whereas in the low MOI, the difference remained at 1 log. After 48 h of incubating process, the regrowth of phage-resistant bacteria in combination with myricetin remained 1 log lower than those of the cells without myricetin treatment at both high and low MOI. It is important to note that in the myricetin treatment group with low MOI (Fig. 3.4b), the viable count remained at 7 log CFU/mL after 3 h of phage addition, consistent with the level before phage addition. Treatment with a high MOI of phage reduced the lysis of the *ΔpriA* mutant strain, resulting in a decline to only 2 log CFU/mL after 3 h of phage addition, and promoted earlier regrowth of *ΔpriA* resistant bacteria, reaching 6.2 log

CFU/mL at 24 h of incubating process (Fig. 3.4a). However, when combined with myricetin treatment, the phage at a high MOI promoted the lysis of the *ΔpriA* mutant strain and delayed the regrowth of *ΔpriA* resistant bacteria within 48 h of incubation.

3.3.4. Impacts of chloramphenicol and phage on the control of viability and regrowth of *E. coli ΔdnaK* mutant strain

To explore the effects of chloramphenicol on phage vulnerability of *ΔdnaK* mutant strain, the strain was added with phage S127BCL3 and 5 μmol/L chloramphenicol together after 6 h incubating process. The MOIs were set to be high and low with the phage S127BCL3 final concentration of 10^9 PFU/mL (MOI=10) and 10^6 PFU/mL (MOI=0.01), respectively. A notable disparity was observed between the high and low MOI (Multiplicity of Infection) groups. When the MOI was high, the viable count in the chloramphenicol treatment group was 1 log lower than in the group without chloramphenicol after 3 and 6 h of phage addition (Fig. 3.5a). Conversely, in the low MOI, the viable count in the chloramphenicol treatment group was 1 log higher than those of the cells without chloramphenicol after 6 h of phage addition (Fig. 3.5b). Whether the MOI was high or low, the viable count in the chloramphenicol treatment group with phage was 1 log higher than those of the cells treated with phage alone after 48 h of incubating process (Fig. 3.4). Treatment with a high MOI of phage reduced the lysis of the *ΔdnaK* mutant strain, resulting in a decline to only 2.2 log CFU/mL after 3 h of phage addition, and promoted earlier regrowth of *ΔdnaK* resistant bacteria, reaching 3.2 log CFU/mL after 6 h of phage addition (Fig. 3.5a). However, when combined with chloramphenicol treatment, the phage at a high MOI promoted the lysis of the *ΔdnaK* mutant strain and delayed the regrowth of *ΔdnaK* resistant bacteria within the first 24 h of incubating process. Therefore, the MOI was set to be high (more than 1) in subsequent experiments.

3.3.5. Impacts of chloramphenicol and phage on the control of viability and regrowth of non-pathogenic *E. coli* BW25113 pretreated with myricetin

To explore the combined effects of myricetin and chloramphenicol on the phage vulnerability of *E. coli* BW25113 (parental strain, wild-type), The strain underwent pretreatment with 500 μmol/L myricetin for 6 h. and then added with 5 μmol/L chloramphenicol and phage S127BCL3 (MOI=1) after the pretreatment. As shown in Fig. 3.6a, the viable counts of the cells cultured with 500 μmol/L myricetin for 48 h showed no significant difference compared to those of the group treated with phage alone. From 3 h after phage inoculation until 24 h, the viable count of *E. coli* exposed to chloramphenicol and phage was approximately 3.2 log CFU/mL without myricetin pretreatment and 2.3 log CFU/mL with myricetin pretreatment. The viable counts of those cells treated with myricetin, chloramphenicol, and phage for 12 and 24 h were 1 log lower (after 6 h from phage introduction) compared to cells treated with phage and chloramphenicol only. However, the counts of cells treated with phage and chloramphenicol were also lower than those with or without myricetin pretreatment after 24 h of incubating process. Although the combined effects of chloramphenicol and phage, with or without myricetin pretreatment, significantly slowed the regrowth of resistant bacterial populations, treatment with chloramphenicol reduced cell lysis, resulting in a viable count of 3.3 log CFU/mL after 3 h of phage addition.

3.3.6. Impacts of chloramphenicol and phage on the control of viability and regrowth of pathogenic *E. coli* O157:H7 pretreated with myricetin

To explore the combined impacts of myricetin and chloramphenicol on the phage vulnerability of pathogenic *E. coli* O157:H7, the strain was pretreated with 500 μmol/L myricetin for 6 h and then added with 5 μmol/L chloramphenicol and phage S127BCL3 (MOI=1) after the pretreatment. The results (Fig. 3.6b) indicated that treatment with chloramphenicol reduced cell lysis, resulting in a viable count of 7.1 log CFU/mL, whereas cells pretreated with myricetin declined to 2 log CFU/mL after 3 h of phage addition. Additionally, the phage S127BCL3 alone was not effective in reducing the pathogenic cells when the phage was introduced to the suspensions after 6 h of growth. However, the viable counts of cells treated with substances and phage for 12 h were 3

logs lower (after 6 h from phage introduction) compared to cells treated with phage alone. After 24 h of incubating process, the viable cell counts of regrowth in the resistant cell groups treated with substances and phage were comparable to those of cells treated with phage alone. Furthermore, after 48 h of incubating process, the viable cell counts of phage-resistant cells in the myricetin pretreated group were 1 log higher than those of cells treated with chloramphenicol and phage but lower than those of cells in the phage alone group. Although the combined effects of myricetin and phage, with or without chloramphenicol, significantly promoted cell lysis, resulting in a viable count of 2 log CFU/mL after 3 h of phage addition, the regrowth of resistant cells was higher with myricetin treatment compared to cells treated with chloramphenicol after 48 h of incubating process. Overall, the combined effects of chloramphenicol and phage, along with myricetin pretreatment, significantly increased cell lysis and slowed the regrowth of phage-resistant pathogenic *E. coli* O157:H7 populations.

3.4. Discussion

Bacteria have developed multiple mechanisms to resist phage infections, which can be categorized into extracellular and intracellular resistance mechanisms. Phage resistance in bacteria can indeed manifest in both external and internal forms, depending on the mechanisms employed by the bacteria to counteract different phages. In this study, the findings revealed that resistant bacteria could be reinfected by the same phage when combined with PriA inhibitor, chloramphenicol (Fig. 3.2). This indicates that the resistance mechanisms of the *E. coli* O157:H7 populations against phage S157 BCL3 are intracellular, as the phage S127BLC3 can still recognize the same receptor in the bacteria, even after they have mutated to resistant forms. The host range of viruses varies significantly, with both specialists and generalists being commonly found in diverse environments. Specialists have a limited host range, infecting only one or a small group of similar hosts, whereas generalists have a wider host range, capable of infecting multiple, distinct types of hosts (Doron et al., 2016). Phage S127BCL3, isolated from chicken liver infected with *E. coli* O157:H7, exhibited

high nucleotide sequence similarity in five out of ten genes encoding tail fiber proteins to *E. coli* phage vB_EcoM-ECP26. This phage, also isolated from *E. coli* O157:H7 and belonging to the *Myoviridae* family, is known for its broad host spectrum (Park et al., 2020). Consequently, phage S127BCL3 demonstrated a wide host range, infecting not only pathogenic *E. coli* O157:H7 but also the non-pathogenic *E. coli* BW25113, classifying it as a generalist phage. The findings align with other research indicating that phage resistance is mainly extracellular when dealing with specialist phages, but shifts to intracellular resistance against generalist phages (Zborowsky and Lindell, 2019).

Chloramphenicol, a well-known hydrophobic bacteriostatic antibiotic, can inhibit protein synthesis in bacteria as mentioned in Chapter 2. In this study, phage-resistant *E. coli* O157:H7 strains were again sensitive to chloramphenicol even at low concentrations without phage infection (Fig. 3.2). Another study reported that Chloramphenicol suppresses the evolution of phage resistance by reducing mutations on the bacterial cell surface (Parab et al., 2023). This research found that phage ΦX174 caused modifications in the lipopolysaccharides (LPS) of the *Escherichia coli* C wildtype strain, leading to phage resistance characterized by "rough" or "deep-rough" LPS phenotypes. Subinhibitory concentrations of chloramphenicol inhibit the growth of deep rough mutants, whereas gentamicin, a hydrophilic antibiotic that also targets protein synthesis, does not show the same inhibition. The combination treatment of ΦX174 and chloramphenicol in *E. coli* C reduces the evolution of phage resistance by eliminating deep rough mutants. The report suggests a high likelihood that some of the phage-resistant mutants in this study were deep rough LPS mutants and were susceptible to chloramphenicol, given that not all the resistant strains' mutations were examined in this study. However, using chloramphenicol alone could not eliminate all the resistant mutants. Combining it with the same phage resulted in greater suppression of the regrowth of the resistant bacteria.

The transfer of genetic material between organisms, known as horizontal gene transfer (HGT), involves the movement of genes within populations in ways other than traditional reproduction. It is a significant mechanism for bacterial evolution, adaptation, and the spread of resistance (Krüger and Stingl, 2011). PriA, a helicase, plays a crucial role in various forms of HGT, particularly in the DNA uptake and integration processes. In natural transformation, bacteria take up naked DNA from their environment. PriA is involved in pulling single-stranded DNA (ssDNA) into the cytoplasm during DNA uptake (Fischer et al., 2020). The helicase activity of PriA helps in unwinding and processing the incoming DNA strand, making it available for recombination and integration into the bacterial genome (Kline and Seifert, 2005). Once the DNA is inside the cell, PriA's role extends to facilitating homologous recombination, a crucial step for integrating the foreign DNA into the host chromosome. This is essential for the maintenance of genetic integrity and the functional expression of acquired genes. While PriA's role is more directly documented in natural transformation, its general involvement in DNA repair and replication restart suggests it might also assist in processes related to conjugation (Golz and Stingl, 2021). During conjugation, the transfer of plasmid DNA requires the stabilization of the replication fork, a process in which PriA is heavily involved. By restarting stalled replication forks, PriA ensures that the transferred DNA is properly replicated and maintained in the recipient cell. PriA’s role in transduction is less direct but can be inferred through its involvement in DNA repair and homologous recombination. Following transduction, the introduced DNA can cause replication fork stalling or DNA damage. PriA is critical in recognizing and repairing these issues, thus facilitating the successful incorporation and maintenance of transduced genes within the host genome. PriA's versatile role in various forms of horizontal gene transfer underscores its essential function in DNA uptake, integration, and repair mechanisms.

DnaK plays a multifaceted role in both bacteriophage lambda replication and DNA repeat rearrangements in *E. coli* (Liberek et al., 1988). During phage infection, DnaK

is essential for initiating replication by modifying the interaction between the phage P protein and the DnaB helicase. In the broader context of the bacterial genome, DnaK facilitates the repair of replication forks through a template-switching mechanism that can lead to deletions or expansions of DNA repeats. This highlights the central importance of DnaK in maintaining the integrity of DNA replication processes under various conditions. The relationship between DnaK and PriA in the context of DNA replication fork repair is examined in another study (Goldfless et al., 2006). The study suggests that the replication fork repair mechanism mediated by DnaK can function independently of PriA. Mutants of DnaK show defects in surviving by replication fork inhibition, but this defect does not seem to require PriA for fork restart. This indicates that the replication fork repair pathway involving DnaK does not necessarily depend on the PriA-dependent fork reassembly mechanism. The study highlights that the fork remains largely intact during the DnaK-dependent repair process, which implies that DnaK can facilitate repair without the need for PriA or PriC restart proteins. This is significant because PriA is typically required to reassemble the fork helicase and DNA polymerase III replication complex after repair. Genetic experiments show that deletion of chromosomal repeats is not dependent on PriA. For example, deletion of repeats was not affected by mutations in PriA or PriC, suggesting that these proteins are not required for the DnaK-mediated repair pathway. In fact, the study found that a priA recA double mutant exhibited a hyperdeletionogenic phenotype, indicating increased genetic instability, which contrasts with the stability provided by DnaK. The researchers propose a crossfork template-switching mechanism for replication fork repair, where DnaK may help to remodel the replication machinery and facilitate the unwinding and realignment of nascent DNA strands. This process can occur without dismantling the fork helicase, bypassing the need for PriA. Overall, the relationship between DnaK and PriA in replication fork repair is characterized by DnaK's ability to mediate a repair process that does not rely on PriA-dependent fork reassembly. This indicates that DnaK plays a crucial role in an alternative repair pathway, enhancing the flexibility and robustness of the cellular response to replication stress.

In Chapter 3, to compare with the myricetin group that required a 6-hour pretreatment, all the cells in the other experimental groups were allowed to grow for the first 6 h before being treated with phage. This method differed from that used in Chapter 2, where the cells were treated with phage from the beginning. Since the bacteria were in the exponential phase, cell lysis by the phage alone was not as efficient as it was during the stationary phase. When phage treatment was combined with chloramphenicol in *E. coli* O157:H7, the speed of cell lysis was slowed by the antibiotic during the first 3 h (Fig. 3.6a). According to a study, bacteriostatic antibiotics inhibit bacterial cell growth without causing cell death, significantly reduce bacterial exponential growth rates and promote the acquisition of CRISPR-Cas immunity by slowing down the replication of phages. This delay allows more time for bacteria to acquire spacers from phages, enhancing their immune response (Dimitriu et al., 2022). Short-term infection experiments show higher rates of CRISPR immunity acquisition in the existence of bacteriostatic antibiotics, while bactericidal antibiotics have no such effect. Bacteriostatic antibiotics delay the production of mature phage particles, thereby extending the opportunity for CRISPR-Cas systems to acquire spacers. Chloramphenicol promotes CRISPR immunity across a broad range of concentrations, suggesting that even low doses can be effective in this regard.

However, pretreatment with myricetin initially led to higher cell lysis by phage within the first 3 h. To significantly reduce phage resistance, A pre-incubation period exceeding 6 h was necessary (Fig. 3.3). The value of the logarithm of the apparent permeability coefficient (LogP_{app}), which indicates hydrophobicity, is high at 1.23 for myricetin, suggesting that these polyphenols exhibit high hydrophobicity (Gonzales et al., 2015). Consequently, the uptake rate of these polyphenols into bacteria is presumed to be very slow, which may explain why myricetin required over 6 h of incubating process to be effective in *E. coli* cells. Myricetin was chosen due to reports indicating its ability to inhibit the cellular function of DnaK (Chang et al., 2011). Flavonoids represent the predominant bioactive compounds found in plant. Researchers are

increasingly investigating plant-derived compounds due to the beneficial properties of phytochemicals like flavonoids, have been shown to disrupt both gram-positive and gram-negative bacteria, targeting essential molecular mechanisms crucial for microbial survival.

3.5. Summary

In summary, the synergistic effects of PriA inhibition and targeting other resistance mechanisms linked to DnaK result in enhanced suppression of regrowth in phage-resistant populations. These findings suggest that employing a combination of phages, flavonoids such as myricetin, and low doses of hydrophobic antibiotics such as chloramphenicol could provide valuable insights for developing effective strategies to control pathogenic *E. coli* O157:H7.

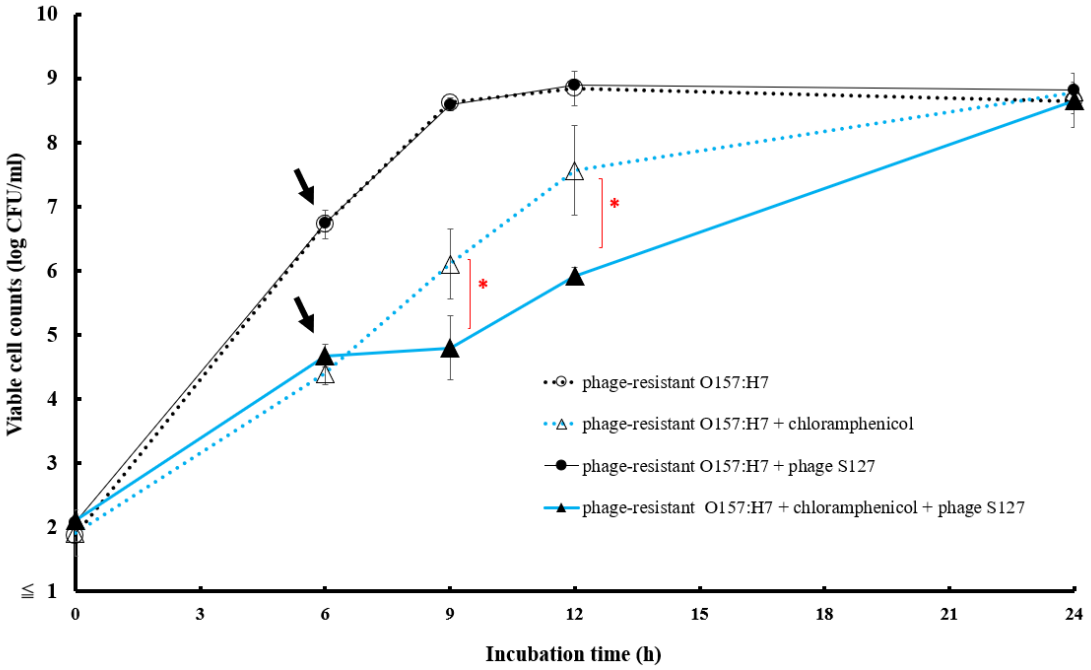


Fig. 3.2. Effects of PriA inhibitor on phage vulnerability of phage-resistant *E. coli* O157:H7 cells. The cells were incubated in LB broth containing 1 mmol/L $CaCl_2$, either in the lack (○, ●) or existence (△, ▲) of 5 μmol/L chloramphenicol. Phage S127BCL3 (MOI=10) was introduced to the culture (●, ▲) after 6 h of incubating process. The viable counts, shown as mean ± S.D. from three separate experiments, are depicted in the graph. The arrow indicates the timing of phage addition.

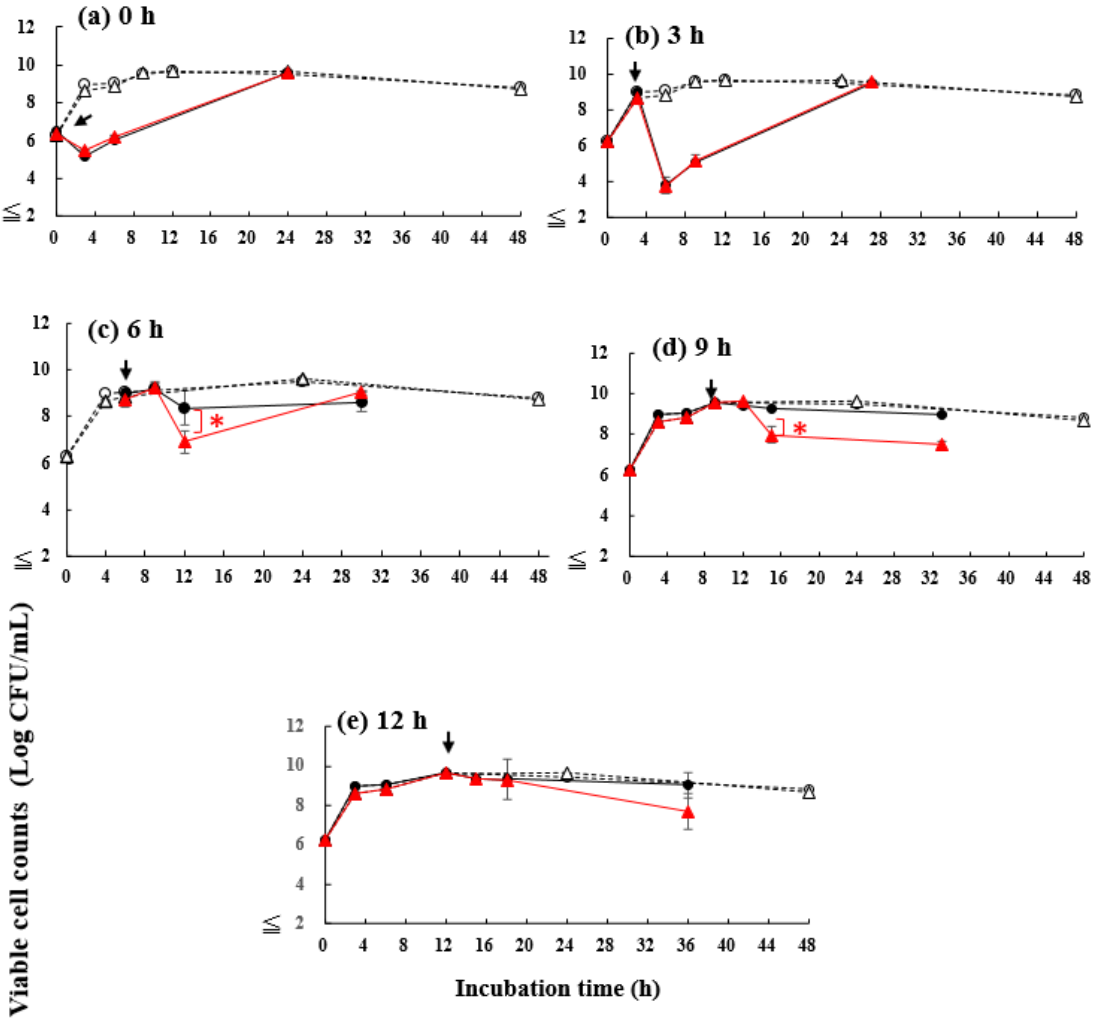
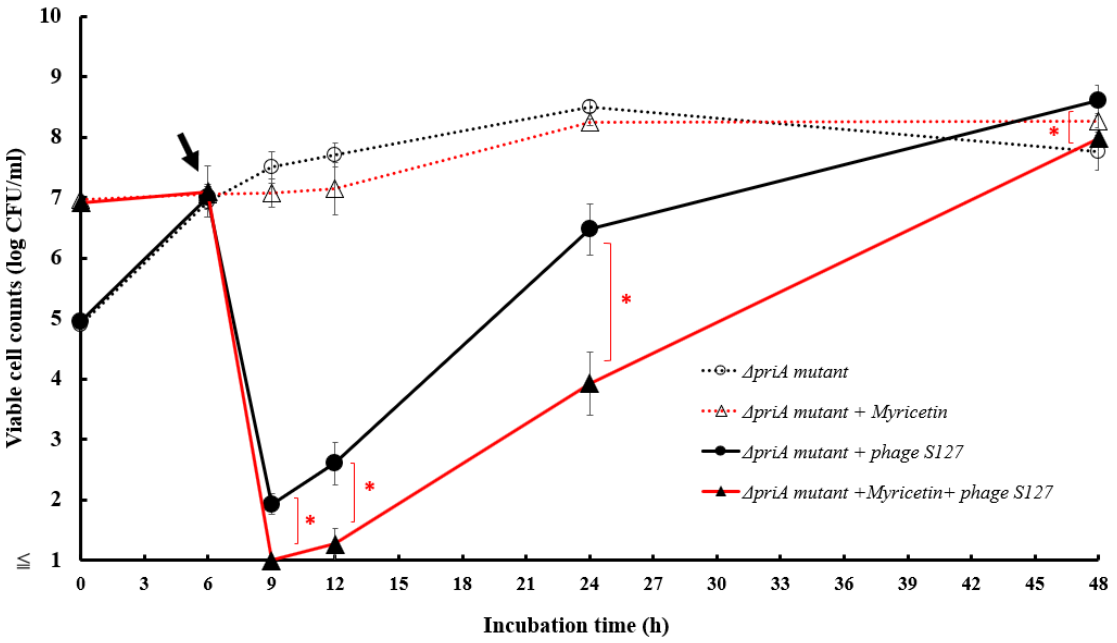


Fig. 3.3. Impact of myricetin treatment duration on the phage vulnerability of *E. coli* BW 25113 cells. The cells were incubated in LB broth containing 1 mmol/L CaCl2, either in the lack (○, ●) or existence (△, ▲) of 500 μmol/L myricetin. Phage S127BCL3 (MOI=1) was introduced to the culture (●, ▲)after 0, 3, 6, 9, and 12 h of incubating process. The arrow indicates the timing of phage addition.

(a) MOI=10



(b) MOI=0.001

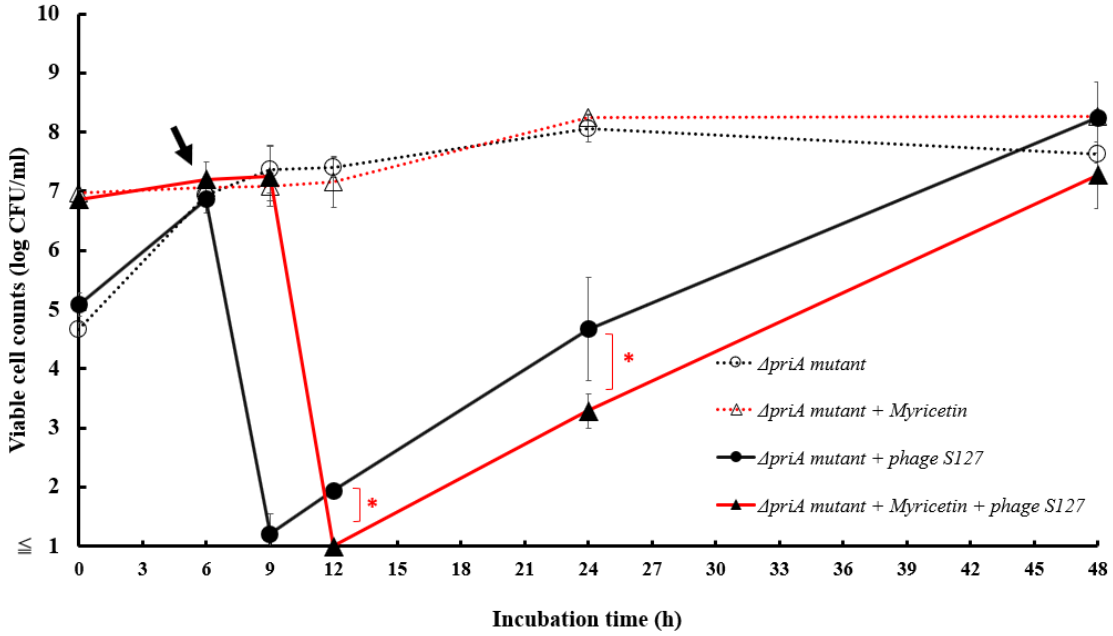
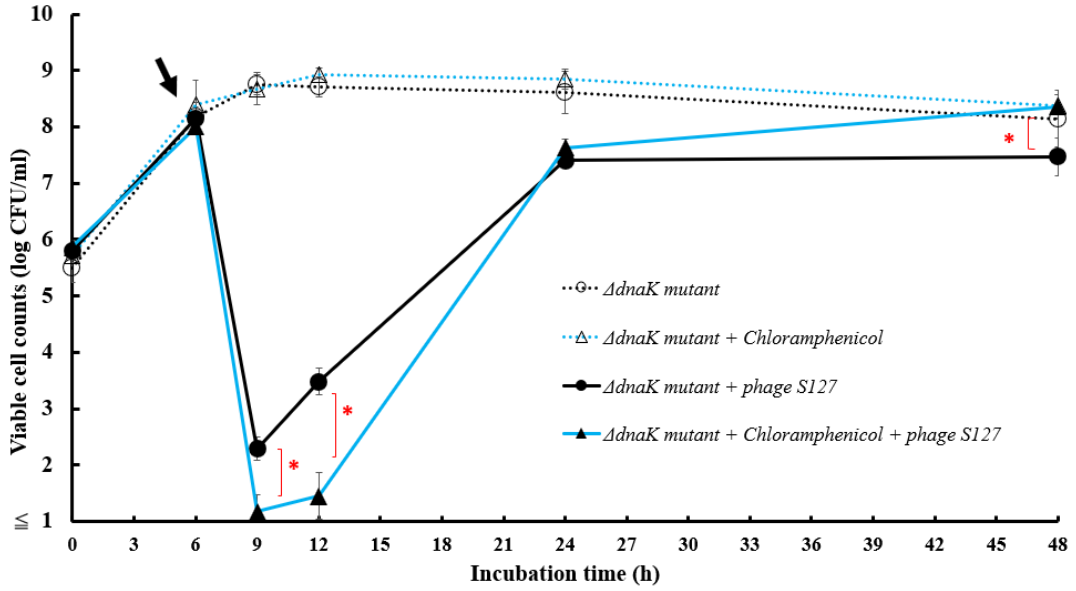


Fig. 3.4. Combined effect of myricetin and phage on the control of viability and regrowth of *E. coli ΔpriA* mutant strain. The cells were incubated in LB broth containing 1 mmol/L $CaCl_2$, either in the lack (○, ●) or existence (△, ▲) of 500 μmol/L myricetin. Phage S127BCL3 (MOI=10 or 0.001) was introduced to the culture (●, ▲) after 6 h of incubating process. Viable counts, represented as mean ± S.D. from three separate experiments, are shown. The arrow indicates the timing of phage addition.

(a) MOI=10



(b) MOI=0.01

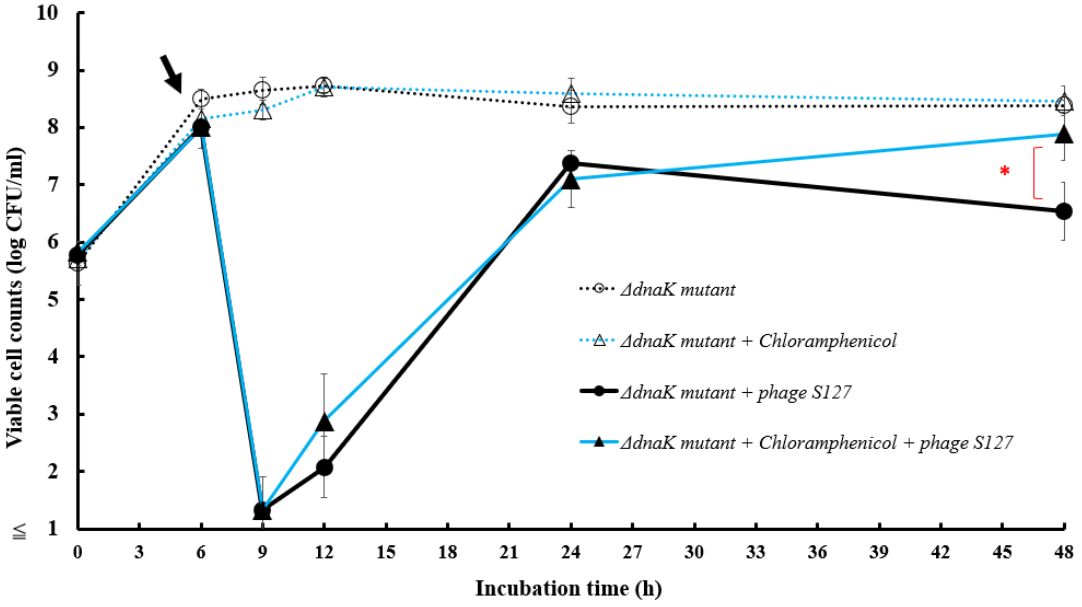
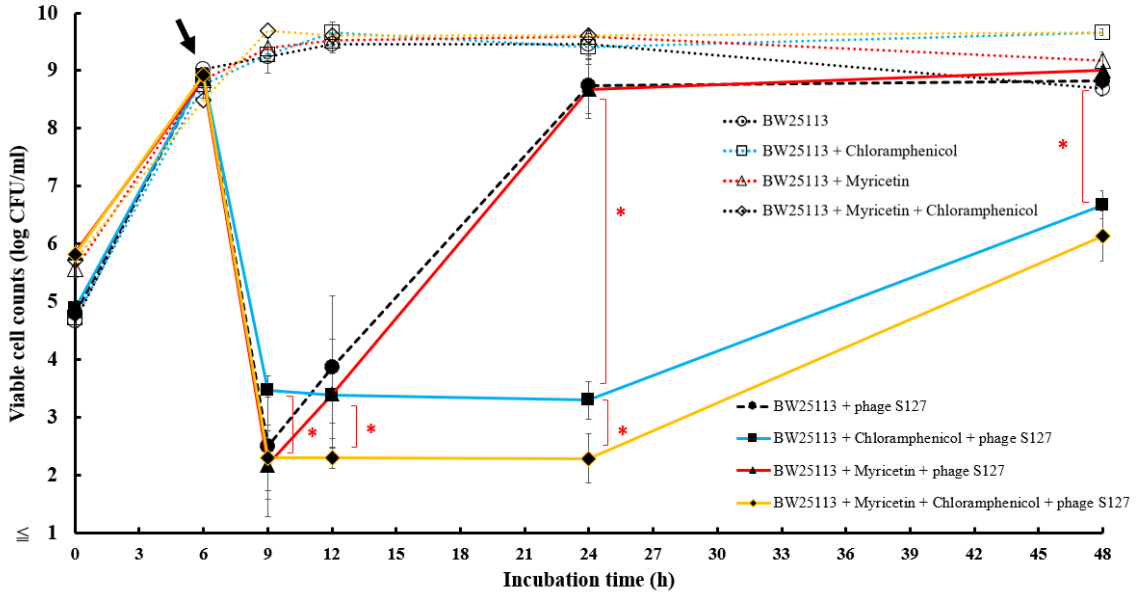


Fig. 3.5. Combined effect of chloramphenicol and phage on the control of viability and regrowth of *E. coli ΔdnaK* mutant strain. The cells were incubated in LB broth containing 1 mmol/L $CaCl_2$. After 6 h of incubating process, 5 µmol/L chloramphenicol was added to the culture (△, ▲). Phage S127BCL3 (MOI=1 or 0.001) was then introduced to the culture either with (▲) or without (●) chloramphenicol. Viable counts, represented as mean ± S.D. from three separate experiments, are shown. The arrow indicates the timing of phage and chloramphenicol addition together.

(a) BW25113



(b) O157:H7

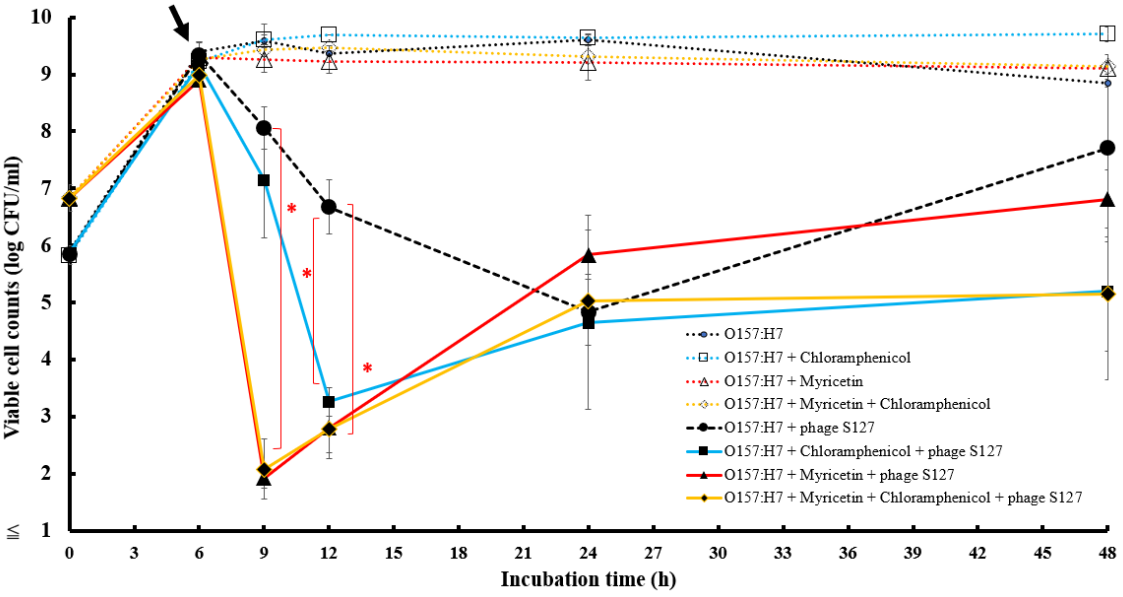


Fig. 3.6. Combined effect of chloramphenicol and phage on the control of viability and regrowth of *E. coli* BW25113 (a) and O157:H7 (b) pretreated with myricetin. *E. coli* was incubated in LB broth containing 1 mmol/L $CaCl_2$, either in the lack (○, ●, □, ■) or existence (△, ▲, ◇, ◆) of 500 μmol/L myricetin. Phage S127BCL3 (MOI=1) was introduced to the culture (●, ▲, ■, ◆) after 6 h of incubating process, either with or without 500 μmol/L myricetin. Viable counts, represented as mean ± S.D. from three separate experiments, are shown. The arrow indicates the timing of phage and chloramphenicol (□, ◇, ■, ◆) addition together.

Chapter 4. Conclusions

Despite the growing attention to phage therapy for bacterial control, its application faces challenges due to the rise of phage-resistant bacterial populations. Numerous countries have approved phage products for food use, highlighting a global consensus on their potential to enhance food safety (Moye et al., 2018). However, the emergence of phage-resistant bacterial isolates presents a significant technical challenge in applying phage biocontrol strategies for food safety enhancement (Hong et al., 2016). To counter phage resistance, researchers have developed various innovative strategies, each with distinct mechanisms and potential applications. Approaches such as deploying phage cocktails, using flavonoids, applying phage-derived enzymes like endolysins, genetically modifying phages, and leveraging phage coevolution have shown promise in overcoming resistance. Ongoing research aims to refine these methods and explore their clinical applications. The choice of strategy depends on the specific bacterial target, infection type, and biocontrol environment. This study aims to investigate a strategic method to improve the effectiveness of phages against foodborne pathogenic *E. coli* O157:H7. It focuses on understanding the genetic basis of resistance within *E. coli* populations, identifying substances that can inhibit functions of resistance-related genes, and examining the combined effects of these substances with phages to enhance phage lytic activity against *E. coli* O157:H7.

Chapter 2 explores the mechanisms of phage resistance in *Escherichia coli*, emphasizing the role of the primosomal protein A (PriA) and its interaction with substances that affect its function. It highlights the importance of understanding these mechanisms to develop effective microbial control strategies. Non-pathogenic *E. coli* strains BW25113, along with 3,909 mutants from the Keio collection, were used for screening phage resistance. Phage S127BCL3 was isolated from chicken livers and tested for lytic activity against *E. coli* BW25113. Substances that inhibit PriA function, such as kanamycin (2 µmol/L and 5 µmol/L) and chloramphenicol (2 µmol/L and 5 µmol/L), significantly increased phage susceptibility in *E. coli* BW25113 and O157:H7.

For example, the viable cell count of *E. coli* BW25113 treated with phage S127BCL3 alone showed a decrease to approximately 10^5 CFU/mL within 6 h and regrowth to 10^9 CFU/mL after 24 h. In contrast, when combined with 5 μmol/L kanamycin, the viable cell count decreased to below 10^2 CFU/mL within 6 h and showed no regrowth for the remaining duration of the experiment (48 h). Similarly, the combination of phage and 5 μmol/L chloramphenicol resulted in a viable cell count of 10^3 CFU/mL at 6 h, with delayed regrowth compared to the phage-only treatment. Notably, in *E. coli* O157:H7, chloramphenicol proved to be more significant than kanamycin in enhancing phage susceptibility. The combination of phage and 5 μmol/L chloramphenicol resulted in a 2-log reduction in bacterial count after 24 h, with delayed regrowth compared to kanamycin. Chloramphenicol's ability to delay the regrowth of phage-resistant cells more effectively than kanamycin highlights its crucial role in the combined treatment strategy. The transcription level of *priA* increased significantly by more than 16-fold after 16 h of phage treatment, indicating its role in phage resistance mechanisms. The results in Chapter 2 elaborate on the significance of PriA in phage resistance and how its inhibition by specific substances can enhance phage susceptibility. The combined action of phages and substances inhibiting PriA function was found to be an effective strategy against pathogenic *E. coli* O157:H*7,* providing insights into potential biocontrol methods to tackle phage-resistant bacteria.

Chapter 3 demonstrated the synergistic effects of inhibiting PriA and DnaK functions using chloramphenicol and myricetin, respectively, to enhance the suppression of phage-resistant *E. coli* populations. Firstly, chloramphenicol significantly increased the phage susceptibility of phage-resistant *E. coli* O157:H7. At 5 μmol/L, chloramphenicol pretreatment reduced the viable cell counts by 2 logs after 6 h compared to untreated controls. The combined effect of chloramphenicol (5 μmol/L) and phage S127BCL3 reinfection (MOI=10) resulted in a 4.8 log reduction in viable cell counts of phage-resistant *E. coli* O157:H7 after 3 h and a 5.9 log reduction after 6 h. Secondly, to explore the effects of myricetin on the phage susceptibility of the *E. coli ΔpriA* mutant strain. 6 h of myricetin pretreatment (500 μmol/L) enhanced the

susceptibility of *E. coli ΔpriA* mutant strains to phage S127BCL3. The viable counts of *E. coli ΔpriA* mutant strains were 1 log lower than untreated controls after 6 h of phage addition. At a high multiplicity of infection (MOI=10), the viable cell count of *E. coli ΔpriA* mutant strains in the myricetin-treated group was 2 logs lower after 24 h and remained 1 log lower after 48 h compared to the group treated with phage alone. To evaluate the effects of chloramphenicol on the phage susceptibility of the *E. coli ΔdnaK* mutant strain. The MOIs were separated to high (MOI=10) and low (MOI=0.01) groups. Viable counts in the chloramphenicol (5 μmol/L) treatment group were 1 log lower than the non-chloramphenicol group after 3 and 6 h of phage addition while MOI being high. In contrast, viable counts in the chloramphenicol treatment group were 1 log higher than the non-chloramphenicol group after 6 h of phage addition while the MOI being low. The chloramphenicol treatment combined with a high MOI phage promoted lysis of the *ΔdnaK* mutant strain and delayed regrowth during the first 24 h of incubation. This demonstrated that chloramphenicol enhances the phage susceptibility of *E. coli ΔdnaK* mutants, particularly under conditions of high MOI, contributing to a significant reduction in viable cell counts of *E. coli ΔdnaK* mutants and delaying the regrowth of phage-resistant bacteria. Finally, the combined treatment of myricetin (500 μmol/L) and chloramphenicol (5 μmol/L) with phage S127BCL3 on *E. coli* BW25113 showed that the viable counts were significantly lower. The *E. coli* BW25113 strain was pretreated with 500 μmol/L myricetin for 6 h, followed by the addition of 5 μmol/L chloramphenicol and phage S127BCL3 (MOI=1). No significant difference was observed between the viable counts of phage-only treatment and those with myricetin after 48 h. However, from 3 to 24 h post-phage inoculation, viable counts were lower in myricetin-pretreated cells (2.3 log CFU/mL) compared to non-pretreated cells (3.2 log CFU/mL). Myricetin, chloramphenicol, and phage treatment reduced viable counts by 1 log more than phage and chloramphenicol alone. Chloramphenicol reduced cell lysis, resulting in a viable count of 3.3 log CFU/mL after 3 h. For pathogenic *E. coli* O157:H7, cells treated with myricetin, chloramphenicol, and phage showed viable counts 3 logs lower compared to those treated with phage alone after 6 h. Viable cell

counts of regrowth in resistant groups treated with substances and phage were similar to those treated with phage alone after 24 h. However, they were still lower than those in the phage alone group after 48 h. The combined treatment of chloramphenicol and phage, along with myricetin pretreatment, significantly promoted cell lysis and slowed the regrowth of phage-resistant pathogenic *E. coli* O157:H7 populations. The study demonstrated that the synergistic combination of myricetin, chloramphenicol, and phages significantly enhances the control of phage-resistant pathogenic *E. coli* O157:H7. This approach increased initial cell lysis and effectively slowed down the regrowth of resistant bacterial populations, suggesting a potent strategy for managing bacterial resistance in food safety applications.

In conclusion, the study demonstrated that targeting PriA with chloramphenicol and DnaK with myricetin leads to enhanced suppression of phage-resistant *E. coli* populations. Chloramphenicol’s effect on PriA inhibition reinstates antibiotic sensitivity in phage-resistant strains, while myricetin's inhibition of DnaK, a key chaperone protein, further weakens bacterial defenses against phages. The synergistic inhibition of PriA and DnaK using chloramphenicol and myricetin, respectively, at concentrations of 5 μmol/L and 500 μmol/L, provides a powerful method for controlling pathogenic *E. coli* O157:H7 strains by significantly reducing their viability and regrowth, highlighting the potential of these inhibitors in improving the efficacy of phage therapies.

Acknowledgment

First and foremost, I would like to express my sincere gratitude to my esteemed supervisor, Professor Takahisa Miyamoto, from the Laboratory of Food Hygienic Chemistry, Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University. His unwavering support and encouragement have been invaluable throughout my academic journey in Japan. It has been a great privilege to conduct research and deepen my understanding of food pathogens under his expert guidance. He has consistently provided patient and insightful direction during challenging experiments and has generously offered numerous opportunities for me to attend conferences, significantly broadening my horizons and enriching my academic experience. In my view, he is not only an eminent scholar but also a mentor who imparts valuable life lessons. His extensive knowledge and profound insights have fueled my passion for this thesis and inspired me to continue pursuing research in this field.

Secondly, I would like to express my heartfelt gratitude to my co-supervisor, Associate Professor Ken-ichi Honjoh. He not only provided me with valuable insights for my experiments but also assisted me in resolving many issues related to the management and use of experimental equipment. I am especially grateful to him for guiding me during my time studying in Japan, enabling me to engage in “Kendo” practice. Learning “Kendo” in Japan has always been a dream of mine.

Additionally, I would like to express my sincere gratitude and deep respect to my co-reviewer, Prof. Dr. Katsumi Doi, from the Laboratory of Microbial Genetics at the Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, for his invaluable assistance and insightful suggestions regarding my research. Furthermore, I would like to extend my deepest gratitude to Assistant Professor Yoshimitsu Masuda. Although we are close in age, he is a remarkably experienced researcher whose guidance has been immensely helpful in the design and analysis of numerous experiments. His insights have continually inspired me to view scientific results from various perspectives. I will also cherish the memorable experience of attending the conference together in Germany.

Finally, I would like to express my gratitude to Ms. Akiko Sato and all the other members of the laboratory. I am very happy to have been classmates with all of you, as you have enriched my life in Japan with colorful memories. Your help, encouragement,

and friendly support have made it possible for me to complete this thesis successfully. Certainly, I extend my heartfelt gratitude to my beloved family and friends in Taiwan. Your steadfast support and frequent messages of care have enabled me to complete my studies with a sense of hope and optimism.

Reference

Aladhadh, M., 2023. A review of modern methods for the detection of foodborne pathogens. Microorganisms 11, 1111. https://doi.org/10.3390/microorganisms11051111

Azam, A.H., Tan, X.-E., Veeranarayanan, S., Kiga, K., Cui, L., 2021. Bacteriophage technology and modern medicine. Antibiotics 10, 999. https://doi.org/10.3390/antibiotics10080999

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L., Mori, H., 2006. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. 2, 2006.0008. https://doi.org/10.1038/msb4100050

Bárdy, P., Pantůček, R., Benešík, M., Doškař, J., 2016. Genetically modified bacteriophages in applied microbiology. J. Appl. Microbiol. 121, 618–633. https://doi.org/10.1111/jam.13207

Bari, Md.L., Yeasmin, S., 2018. Chapter 8 - Foodborne diseases and responsible agents, in: Grumezescu, A.M., Holban, A.M. (Eds.), Food Safety and Preservation. Academic Press, pp. 195–229. https://doi.org/10.1016/B978-0-12-814956-0.00008-1

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A., Horvath, P., 2007. CRISPR provides acquired resistance against viruses in prokaryotes. Science 315, 1709–1712. https://doi.org/10.1126/science.1138140

Bintsis, T., 2017. Foodborne pathogens. AIMS Microbiol. 3, 529–563. https://doi.org/10.3934/microbiol.2017.3.529

Blower, T.R., Evans, T.J., Przybilski, R., Fineran, P.C., Salmond, G.P.C., 2012a. Viral evasion of a bacterial suicide system by RNA-based molecular mimicry enables infectious altruism. PLoS Genet. 8, e1003023. https://doi.org/10.1371/journal.pgen.1003023

Blower, T.R., Evans, T.J., Przybilski, R., Fineran, P.C., Salmond, G.P.C., 2012b. Viral evasion of a bacterial suicide system by RNA-based molecular mimicry enables infectious altruism. PLoS Genet. 8, e1003023. https://doi.org/10.1371/journal.pgen.1003023

Borin, J.M., Avrani, S., Barrick, J.E., Petrie, K.L., Meyer, J.R., 2021. Coevolutionary phage training leads to greater bacterial suppression and delays the evolution of phage resistance. Proc. Natl. Acad. Sci. 118, e2104592118. https://doi.org/10.1073/pnas.2104592118

Borin, J.M., Lee, J., Gerbino, K.R., Meyer, J.R., 2022. Comparison of bacterial suppression by phage cocktails, dual-receptor generalists, and coevolutionarily trained phages. Evol. Appl. 16, 152–162. https://doi.org/10.1111/eva.13518

Bulkley, D., Innis, C.A., Blaha, G., Steitz, T.A., 2010. Revisiting the structures of several antibiotics bound to the bacterial ribosome. Proc. Natl. Acad. Sci. 107, 17158–17163. https://doi.org/10.1073/pnas.1008685107

Burmeister, A.R., Fortier, A., Roush, C., Lessing, A.J., Bender, R.G., Barahman, R., Grant, R., Chan, B.K., Turner, P.E., 2020. Pleiotropy complicates a trade-off between phage resistance and antibiotic resistance. Proc. Natl. Acad. Sci. U. S. A. 117, 11207–11216. https://doi.org/10.1073/pnas.1919888117

Cenci-Goga, B.T., Karama, M., Sechi, P., Iulietto, M.F., Novelli, S., Mattei, S., 2014. Evolution under different storage conditions of anomalous blue coloration of Mozzarella cheese intentionally contaminated with a pigment-producing strain of *Pseudomonas fluorescens*. J. Dairy Sci. 97, 6708–6718. https://doi.org/10.3168/jds.2014-8611

Cesa, L.C., Patury, S., Komiyama, T., Ahmad, A., Zuiderweg, E.R.P., Gestwicki, J.E., 2013. Inhibitors of difficult protein–protein interactions identified by high-throughput screening of multiprotein complexes. ACS Chem. Biol. 8, 1988–1997. https://doi.org/10.1021/cb400356m

Chang, L., Miyata, Y., Ung, P.M.U., Bertelsen, E.B., McQuade, T.J., Carlson, H.A., Zuiderweg, E.R.P., Gestwicki, J.E., 2011. Chemical screens against a reconstituted multiprotein complex: myricetin blocks dnaJ regulation of DnaK through an allosteric mechanism. Chem. Biol. 18, 210–221. https://doi.org/10.1016/j.chembiol.2010.12.010

Christiansen, R.H., Madsen, L., Dalsgaard, I., Castillo, D., Kalatzis, P.G., Middelboe, M., 2016. Effect of Bacteriophages on the growth of flavobacterium psychrophilum and development of phage-resistant strains. Microb. Ecol. 71, 845–859. https://doi.org/10.1007/s00248-016-0737-5

Chulluncuy, R., Espiche, C., Nakamoto, J.A., Fabbretti, A., Milón, P., 2016. Conformational response of 30S-bound IF3 to a-site binders streptomycin and kanamycin. Antibiotics 5, 38. https://doi.org/10.3390/antibiotics5040038

Clarke, S.C., 2001. Diarrhoeagenic *Escherichia coli*—an emerging problem? Diagn. Microbiol. Infect. Dis. 41, 93–98. https://doi.org/10.1016/S0732-8893(01)00303-0

Comeau, A.M., Tétart, F., Trojet, S.N., Prère, M.-F., Krisch, H.M., 2007. Phage-antibiotic synergy (PAS): beta-lactam and quinolone antibiotics stimulate virulent phage growth. PloS One 2, e799. https://doi.org/10.1371/journal.pone.0000799

Cushnie, T.P.T., Lamb, A.J., 2005. Antimicrobial activity of flavonoids. Int. J. Antimicrob. Agents 26, 343–356. https://doi.org/10.1016/j.ijantimicag.2005.09.002

Dalmasso, M., Strain, R., Neve, H., Franz, C., Cousin, F.J., Ross, R., Hill, C., 2016. Three new *Escherichia coli* Phages from the human gut show promising potential for Phage Therapy. PLoS ONE 11. https://doi.org/10.1371/journal.pone.0156773

De Jonge, J., Frewer, L., Van Trijp, H., Jan Renes, R., De Wit, W., Timmers, J., 2004. Monitoring consumer confidence in food safety: an exploratory study. Br. Food J. 106, 837–849.

Deep, A., Liang, Q., Enustun, E., Pogliano, J., Corbett, K.D., 2024. Architecture and infection-sensing mechanism of the bacterial PARIS defense system. BioRxiv Prepr. Serv. Biol. 2024.01.02.573835. https://doi.org/10.1101/2024.01.02.573835

Dimitriu, T., Kurilovich, E., Łapińska, U., Severinov, K., Pagliara, S., Szczelkun, M.D., Westra, E.R., 2022. Bacteriostatic antibiotics promote CRISPR-Cas adaptive immunity by enabling increased spacer acquisition. Cell Host Microbe 30, 31-40.e5. https://doi.org/10.1016/j.chom.2021.11.014

Dontorou, C., Papadopoulou, C., Filioussis, G., Economou, V., Apostolou, I., Zakkas, G., Salamoura, A., Kansouzidou, A., Levidiotou, S., 2003. Isolation of *Escherichia coli* O157: H7 from foods in Greece. Int. J. Food Microbiol. 82, 273–279.

Doron, S., Fedida, A., Hernández-Prieto, M.A., Sabehi, G., Karunker, I., Stazic, D., Feingersch, R., Steglich, C., Futschik, M., Lindell, D., Sorek, R., 2016. Transcriptome dynamics of a broad host-range cyanophage and its hosts. ISME J. 10, 1437–1455. https://doi.org/10.1038/ismej.2015.210

Doyle, M.P., 1991. *Escherichia coli* O157:H7 and its significance in foods. Int. J. Food Microbiol. 12, 289–301. https://doi.org/10.1016/0168-1605(91)90143-d

Egido, J.E., Costa, A.R., Aparicio-Maldonado, C., Haas, P.-J., Brouns, S.J.J., 2022. Mechanisms and clinical importance of bacteriophage resistance. FEMS Microbiol. Rev. 46, fuab048. https://doi.org/10.1093/femsre/fuab048

Endersen, L., Coffey, A., 2020. The use of bacteriophages for food safety. Current Opinion in Food Science, Food Safety 36, 1–8. https://doi.org/10.1016/j.cofs.2020.10.006

Farooq, T., Hussain, M.D., Shakeel, M., Tariqjaveed, M., Aslam, M.N., Naqvi, S., Amjad, R., Tang, Y., She, X., He, Z., 2022. Deploying viruses against phytobacteria: potential use of phage cocktails as a multifaceted approach to

combat resistant bacterial plant pathogens. Viruses 14. https://doi.org/10.3390/v14020171

Fischer, W., Tegtmeyer, N., Stingl, K., Backert, S., 2020. Four chromosomal type IV secretion systems in *Helicobacter pylori*: composition, structure and function. Front. Microbiol. 11. https://doi.org/10.3389/fmicb.2020.01592

Fister, S., Robben, C., Witte, A.K., Schoder, D., Wagner, M., Rossmanith, P., 2016. Influence of environmental factors on phage–bacteria interaction and on the efficacy and infectivity of phage P100. Front. Microbiol. 7, 1152. https://doi.org/10.3389/fmicb.2016.01152

Folimonova, S.Y., 2012. Superinfection exclusion is an active virus-controlled function that requires a specific viral protein. J. Virol. 86, 5554–5561. https://doi.org/10.1128/JVI.00310-12

Furukawa, I., Suzuki, M., Masaoka, T., Nakajima, N., Mitani, E., Tasaka, M., Teranishi, H., Matsumoto, Y., Koizumi, M., Ogawa, A., Oota, Y., Homma, S., Sasaki, K., Satoh, H., Sato, K., Muto, S., Anan, Y., Kuroki, T., 2018. Outbreak of enterohemorrhagic *Escherichia coli* O157:H7 infection associated with minced meat gutlets consumption in Kanagawa, Japan. Jpn. J. Infect. Dis. 71, 436–441. https://doi.org/10.7883/yoken.JJID.2017.495

Gallo, M., Ferrara, L., Calogero, A., Montesano, D., Naviglio, D., 2020. Relationships between food and diseases: What to know to ensure food safety. Food Res. Int. 137, 109414. https://doi.org/10.1016/j.foodres.2020.109414

Gambushe, S.M., Zishiri, O.T., Zowalaty, M.E.E., 2022. Review of *Escherichia coli* O157:H7 prevalence, pathogenicity, heavy metal and antimicrobial resistance, African Perspective. Infect. Drug Resist. 15, 4645–4673. https://doi.org/10.2147/IDR.S365269

Gencay, Y.E., Jasinskytė, D., Robert, C., Semsey, S., Martínez, V., Petersen, A.Ø., Brunner, K., de Santiago Torio, A., Salazar, A., Turcu, I.C., Eriksen, M.K., Koval, L., Takos, A., Pascal, R., Schou, T.S., Bayer, L., Bryde, T., Johansen, K.C., Bak, E.G., Smrekar, F., Doyle, T.B., Satlin, M.J., Gram, A., Carvalho, J., Jessen, L., Hallström, B., Hink, J., Damholt, B., Troy, A., Grove, M., Clube, J., Grøndahl, C., Haaber, J.K., van der Helm, E., Zdravkovic, M., Sommer, M.O.A., 2024. Engineered phage with antibacterial CRISPR–Cas selectively reduce *E. coli* burden in mice. Nat. Biotechnol. 42, 265–274. https://doi.org/10.1038/s41587-023-01759-y

Genevaux, P., Georgopoulos, C., Kelley, W.L., 2007. The Hsp70 chaperone machines of *Escherichia coli*: a paradigm for the repartition of chaperone functions. Mol. Microbiol. 66, 840–857. https://doi.org/10.1111/j.1365-2958.2007.05961.x

Goldfless, S.J., Morag, A.S., Belisle, K.A., Sutera, V.A., Lovett, S.T., 2006. DNA repeat rearrangements mediated by DnaK-dependent replication fork repair. Mol. Cell 21, 595–604. https://doi.org/10.1016/j.molcel.2006.01.025

Golz, J.C., Stingl, K., 2021. Natural competence and horizontal gene transfer in *Campylobacter*. Curr. Top. Microbiol. Immunol. 431, 265–292. https://doi.org/10.1007/978-3-030-65481-8_10

Gonzales, G.B., Van Camp, J., Zotti, M., Kobayashi, V., Grootaert, C., Raes, K., Smagghe, G., 2015. Two- and three-dimensional quantitative structure–permeability relationship of flavonoids in Caco-2 cells using stepwise multiple linear regression (SMLR), partial least squares regression (PLSR), and pharmacophore (GALAHAD)-based comparative molecular similarity index analysis (COMSIA). Med. Chem. Res. 24, 1696–1706. https://doi.org/10.1007/s00044-014-1241-4

Gordeeva, J., Morozova, N., Sierro, N., Isaev, A., Sinkunas, T., Tsvetkova, K., Matlashov, M., Truncaitė, L., Morgan, R.D., Ivanov, N.V., Siksnys, V., Zeng, L., Severinov, K., 2019. BREX system of *Escherichia coli* distinguishes self from non-self by methylation of a specific DNA site. Nucleic Acids Res. 47, 253–265. https://doi.org/10.1093/nar/gky1125

Goyal, A., 2022. Horizontal gene transfer drives the evolution of dependencies in bacteria. iScience 25, 104312. https://doi.org/10.1016/j.isci.2022.104312

Greer, S., Perham, R.N., 1986. Glutathione reductase from *Escherichia coli*: cloning and sequence analysis of the gene and relationship to other flavoprotein disulfide oxidoreductases. Biochemistry 25, 2736–2742. https://doi.org/10.1021/bi00357a069

Guo, D., Chen, J., Zhao, X., Luo, Y., Jin, M., Fan, F., Park, C., Yang, X., Sun, C., Yan, J., Chen, W., Liu, Z., 2021. Genetic and chemical engineering of phages for controlling multidrug-resistant bacteria. Antibiotics 10. https://doi.org/10.3390/antibiotics10020202

Guo, H., Arambula, D., Ghosh, P., Miller, J.F., 2014. Diversity-generating retroelements in phage and bacterial genomes. Microbiol. Spectr. 2, 10.1128/microbiolspec.MDNA3-0029–2014. https://doi.org/10.1128/microbiolspec.MDNA3-0029-2014

Gupta, A., Peng, S., Leung, C.Y., Borin, J.M., Medina, S.J., Weitz, J.S., Meyer, J.R., 2022. Leapfrog dynamics in phage-bacteria coevolution revealed by joint analysis of cross-infection phenotypes and whole genome sequencing. Ecol. Lett. 25, 876–888. https://doi.org/10.1111/ele.13965

Hashemolhosseine, S., Holmes, Z., Mutschler, B., Henning, U., 1994. Alterations of receptor specificities of coliphages of the T2 family. J. Mol. Biol. 240, 105–110. https://doi.org/10.1006/jmbi.1994.1424

Helmy, Y.A., El-Adawy, H., Sanad, Y.M., Ghanem, M., 2023. Editorial: Food safety and public health. Front. Microbiol. 14, 1169139. https://doi.org/10.3389/fmicb.2023.1169139

Hesse, S.E., Rajaure, M., Wall, E.A., Johnson, J.M., Bliskovsky, V., Gottesman, S., Adhya, S., 2020. Phage resistance in multidrug-resistant *Klebsiella pneumoniae* ST258 evolves via diverse mutations that culminate in impaired adsorption. mBio 11. https://doi.org/10.1128/mBio.02530-19

Hesterkamp, T., Bukau, B., 1998. Role of the DnaK and HscA homologs of Hsp70 chaperones in protein folding in *E.coli*. EMBO J. 17, 4818–4828. https://doi.org/10.1093/emboj/17.16.4818

Hille, F., Richter, H., Wong, S.P., Bratovič, M., Ressel, S., Charpentier, E., 2018. The biology of CRISPR-Cas: backward and forward. Cell 172, 1239–1259. https://doi.org/10.1016/j.cell.2017.11.032

Hong, Y., Schmidt, K., Marks, D., Hatter, S., Marshall, A., Albino, L., Ebner, P., 2016. Treatment of *Salmonella*-contaminated eggs and pork with a broad-spectrum, single bacteriophage: assessment of efficacy and resistance development. Foodborne Pathog. Dis. 13, 679–688. https://doi.org/10.1089/fpd.2016.2172

Huang, Y.-H., Huang, C.-C., Chen, C.-C., Yang, K.-J., Huang, C.-Y., 2015. Inhibition of *Staphylococcus aureus* PriA helicase by flavonol kaempferol. Protein J. 34, 169–172. https://doi.org/10.1007/s10930-015-9609-y

Isaev, A., Drobiazko, A., Sierro, N., Gordeeva, J., Yosef, I., Qimron, U., Ivanov, N.V., Severinov, K., 2020. Phage T7 DNA mimic protein Ocr is a potent inhibitor of BREX defence. Nucleic Acids Res. 48, 5397–5406. https://doi.org/10.1093/nar/gkaa290

Iulietto, M.F., Sechi, P., Borgogni, E., Cenci-Goga, B.T., 2015. Meat spoilage: A critical review of a neglected alteration due to ropy slime producing bacteria. Ital. J. Anim. Sci. 14, 4011. https://doi.org/10.4081/ijas.2015.4011

Ivančić-Baće, I., Cass, S.D., Wearne, S.J., Bolt, E.L., 2015. Different genome stability proteins underpin primed and naïve adaptation in *E. coli* CRISPR-Cas immunity. Nucleic Acids Res. 43, 10821–10830. https://doi.org/10.1093/nar/gkv1213

Júnior, S.D.D.C., Santos, J.V.D.O., Campos, L.A.D.A., Pereira, M.A., Magalhães, N.S.S., Cavalcanti, I.M.F., 2018. Antibacterial and antibiofilm activities of

quercetin against clinical isolates of *Staphyloccocus aureus* and *Staphylococcus saprophyticus* with resistance profile. Int. J. Environ. Agric. Biotechnol. 3, 1948–1958. https://doi.org/10.22161/ijeab/3.5.50

Jurado, A., Fernández, L., Rodríguez, A., García, P., 2022. Understanding the mechanisms that drive phage resistance in *Staphylococci* to prevent phage therapy failure. Viruses 14. https://doi.org/10.3390/v14051061

Karmali, M.A., Gannon, V., Sargeant, J.M., 2010. Verocytotoxin-producing *Escherichia coli* (VTEC). Vet. Microbiol., Zoonoses: Advances and Perspectives 140, 360–370. https://doi.org/10.1016/j.vetmic.2009.04.011

Kazi, M., Annapure, U.S., 2016. Bacteriophage biocontrol of foodborne pathogens. J. Food Sci. Technol. 53, 1355–1362. https://doi.org/10.1007/s13197-015-1996-8

Khan, F.M., Chen, J.-H., Zhang, R., Liu, B., 2023. A comprehensive review of the applications of bacteriophage-derived endolysins for foodborne bacterial pathogens and food safety: recent advances, challenges, and future perspective. Front. Microbiol. 14. https://doi.org/10.3389/fmicb.2023.1259210

Killelea, T., Dimude, J.U., He, L., Stewart, A.L., Kemm, F.E., Radovčić, M., Ivančić-Baće, I., Rudolph, C.J., Bolt, E.L., 2023. Cas1-Cas2 physically and functionally interacts with DnaK to modulate CRISPR adaptation. Nucleic Acids Res. 51, 6914–6926. https://doi.org/10.1093/nar/gkad473

Kline, K.A., Seifert, H.S., 2005. Mutation of the *priA* gene of *Neisseria gonorrhoeae* affects DNA transformation and DNA repair. J. Bacteriol. 187, 5347–5355. https://doi.org/10.1128/JB.187.15.5347-5355.2005

Krüger, N.-J., Stingl, K., 2011. Two steps away from novelty--principles of bacterial DNA uptake. Mol. Microbiol. 80, 860–867. https://doi.org/10.1111/j.1365-2958.2011.07647.x

Kumar, S., Pandey, A.K., 2013. Chemistry and biological activities of flavonoids: an overview. Sci. World J. 2013, 162750. https://doi.org/10.1155/2013/162750

Labrie, S.J., Samson, J.E., Moineau, S., 2010. Bacteriophage resistance mechanisms. Nat. Rev. Microbiol. 8, 317–327. https://doi.org/10.1038/nrmicro2315

Lee, E.H., Masai, H., Allen, G.C., Kornberg, A., 1990. The *priA* gene encoding the primosomal replicative n' protein of *Escherichia coli*. Proc. Natl. Acad. Sci. 87, 4620–4624. https://doi.org/10.1073/pnas.87.12.4620

Lee, H., Yoon, Y., 2021. Etiological agents implicated in foodborne illness world wide. Food Sci. Anim. Resour. 41, 1–7. https://doi.org/10.5851/kosfa.2020.e75

Lee, P.T., Hsu, A.Y., Ha, H.T., Clarke, C.F., 1997. A C-methyltransferase involved in both ubiquinone and menaquinone biosynthesis: isolation and identification of the *Escherichia coli ubiE* gene. J. Bacteriol. 179, 1748–1754. https://doi.org/10.1128/jb.179.5.1748-1754.1997

Leroux, M., Jani, N., Sandler, S.J., 2017. A *priA* mutant expressed in two pieces has almost full activity in *Escherichia coli* K-12. J. Bacteriol. 199, e00267-17. https://doi.org/10.1128/JB.00267-17

Li, N., Zeng, Y., Wang, M., Bao, R., Chen, Y., Li, X., Pan, J., Zhu, T., Hu, B., Tan, D., 2022. Characterization of phage resistance and their impacts on bacterial fitness in *Pseudomonas aeruginosa*. Microbiol. Spectr. 10. https://doi.org/10.1128/spectrum.02072-22

Li, Y., Ding, Y., 2012. Minireview: Therapeutic potential of myricetin in diabetes mellitus. Food Sci. Hum. Wellness 1, 19–25. https://doi.org/10.1016/j.fshw.2012.08.002

Liberek, K., Georgopoulos, C., Zylicz, M., 1988. Role of the *Escherichia coli* DnaK and DnaJ heat shock proteins in the initiation of bacteriophage lambda DNA replication. Proc. Natl. Acad. Sci. U. S. A. 85, 6632–6636.

Lorenzo, J.M., Munekata, P.E., Dominguez, R., Pateiro, M., Saraiva, J.A., Franco, D., 2018. Main groups of microorganisms of relevance for food safety and stability. Innov. Technol. Food Preserv. 53–107. https://doi.org/10.1016/B978-0-12-811031-7.00003-0

Lyngstadaas, A., Sprenger, G.A., Boye, E., 1998. Impaired growth of an *Escherichia coli rpe* mutant lacking ribulose-5-phosphate epimerase activity. Biochim. Biophys. Acta 1381, 319–330. https://doi.org/10.1016/s0304-4165(98)00046-4

Manning, A.J., Kuehn, M.J., 2011. Contribution of bacterial outer membrane vesicles to innate bacterial defense. BMC Microbiol. 11, 258. https://doi.org/10.1186/1471-2180-11-258

Masai, H., Arai, K., 1996. DnaA- and PriA-dependent primosomes: two distinct replication complexes for replication of *Escherichia coli* chromosome. Front. Biosci. J. Virtual Libr. 1, d48-58. https://doi.org/10.2741/a115

Masai, H., Asai, T., Kubota, Y., Arai, K., Kogoma, T., 1994. *Escherichia coli* PriA protein is essential for inducible and constitutive stable DNA replication. EMBO J. 13, 5338–5345. https://doi.org/10.1002/j.1460-2075.1994.tb06868.x

Mayer, M.P., Bukau, B., 2005. Hsp70 chaperones: Cellular functions and molecular mechanism. Cell. Mol. Life Sci. 62, 670. https://doi.org/10.1007/s00018-004-4464-6

Mayer, M.P., Rüdiger, S., Bukau, B., 2000. Molecular basis for interactions of the DnaK chaperone with substrates. Biol Chem 381, 877–885. https://doi.org/10.1515/BC.2000.109

McKenzie, A.M., Henry, C., Myers, K.S., Place, M.M., Keck, J.L., 2022. Identification of genetic interactions with priB links the PriA/PriB DNA replication restart pathway to double-strand DNA break repair in *Escherichia coli*. G3 (Bethesda) 12, jkac295. https://doi.org/10.1093/g3journal/jkac295

McKitterick, A.C., Seed, K.D., 2018. Anti-phage islands force their target phage to directly mediate island excision and spread. Nat. Commun. 9, 2348. https://doi.org/10.1038/s41467-018-04786-5

Mendoza, S.D., Nieweglowska, E.S., Govindarajan, S., Leon, L.M., Berry, J.D., Tiwari, A., Chaikeeratisak, V., Pogliano, J., Agard, D.A., Bondy-Denomy, J., 2020. A bacteriophage nucleus-like compartment shields DNA from CRISPR nucleases. Nature 577, 244–248. https://doi.org/10.1038/s41586-019-1786-y

Michel, B., Sandler, S.J., 2017. Replication restart in bacteria. J. Bacteriol. 199, e00102-17. https://doi.org/10.1128/JB.00102-17

Morrison, P.M., Balmforth, M.R., Ness, S.W., Williamson, D.J., Rugen, M.D., Turnbull, W.B., Webb, M.E., 2016. Confirmation of a protein–protein interaction in the pantothenate biosynthetic pathway by using sortase-mediated labelling. ChemBioChem 17, 753–758. https://doi.org/10.1002/cbic.201500547

Moye, Z.D., Woolston, J., Sulakvelidze, A., 2018. Bacteriophage applications for food production and processing. Viruses 10, 205. https://doi.org/10.3390/v10040205

Mutalik, V.K., Adler, B., Rishi, H.S., Piya, D., Zhong, C., Koskella, B., Calendar, R., Novichkov, P., Price, M., Deutschbauer, A., Arkin, A., 2020. High-throughput mapping of the phage resistance landscape in *E. coli*. PLoS Biol. 18. https://doi.org/10.1101/2020.02.15.951020

Nguyen, Y., Sperandio, V., 2012. Enterohemorrhagic *E. coli* (EHEC) pathogenesis. Front. Cell. Infect. Microbiol. 2, 90. https://doi.org/10.3389/fcimb.2012.00090

North, O.I., Brown, E., 2020. Phage–antibiotic combinations: a promising approach to constrain resistance evolution in bacteria. Ann. N. Y. Acad. Sci. 1496. https://doi.org/10.1111/nyas.14533

Ofir, G., Melamed, S., Sberro, H., Mukamel, Z., Silverman, S., Yaakov, G., Doron, S., Sorek, R., 2018. DISARM is a widespread bacterial defence system with broad anti-phage activities. Nat. Microbiol. 3, 90–98. https://doi.org/10.1038/s41564-017-0051-0

Ong, K.C., Khoo, H.E., 1997. Biological effects of myricetin. Gen. Pharmacol. 29, 121–126. https://doi.org/10.1016/s0306-3623(96)00421-1

Parab, L., Dherbey, J.R., Rivera, N., Schwarz, M., Bertels, F., 2023. Chloramphenicol reduces phage resistance evolution by suppressing bacterial cell surface mutants. *bioRxiv*. https://doi.org/10.1101/2023.08.28.552763

Park, D.-W., Lim, G., Lee, Y., Park, J.-H., 2020. Characteristics of lytic phage vB_EcoM-ECP26 and reduction of shiga-toxin producing *Escherichia coli* on produce romaine. Appl. Biol. Chem. 63, 19. https://doi.org/10.1186/s13765-020-00502-4

Percival, S.L., Williams, D.W., 2014. Chapter Six - Escherichia coli, in: Percival, S.L., Yates, M.V., Williams, D.W., Chalmers, R.M., Gray, N.F. (Eds.), Microbiology of waterborne diseases (Second Edition). Academic Press, London, pp. 89–117. https://doi.org/10.1016/B978-0-12-415846-7.00006-8

Puligundla, P., Lim, S., 2022. Biocontrol Approaches against *Escherichia coli* O157:H7 in foods. Foods 11, 756. https://doi.org/10.3390/foods11050756

Saeedi, P., Yazdanparast, M., Behzadi, E., Salmanian, A.H., Mousavi, S.L., Nazarian, S., Amani, J., 2017. A review on strategies for decreasing *E. coli* O157:H7 risk in animals. Microb. Pathog. 103, 186–195. https://doi.org/10.1016/j.micpath.2017.01.001

Sandler, S.J., Marians, K.J., 2000. Role of PriA in replication fork reactivation in *Escherichia coli*. J. Bacteriol. 182, 9–13.

Sarhan, W.A., Azzazy, H.M., 2015. Phage approved in food, why not as a therapeutic? Expert Rev. Anti Infect. Ther. 13, 91–101. https://doi.org/10.1586/14787210.2015.990383

Sato, M., Murakami, K., Uno, M., Nakagawa, Y., Katayama, S., Akagi, K., Masuda, Y., Takegoshi, K., Irie, K., 2013. Site-specific inhibitory mechanism for amyloid β42 aggregation by catechol-type flavonoids targeting the Lys residues. J. Biol. Chem. 288, 23212–23224. https://doi.org/10.1074/jbc.M113.464222

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.-A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the united states—major pathogens. Emerg. Infect. Dis. 17, 7–15. https://doi.org/10.3201/eid1701.P11101

Sperandio, V., Nguyen, Y., 2012. Enterohemorrhagic *E. coli* (EHEC) pathogenesis. Front. Cell. Infect. Microbiol. 2. https://doi.org/10.3389/fcimb.2012.00090

Tan, Z., Chen, J., Zhang, X., 2016. Systematic engineering of pentose phosphate pathway improves *Escherichia coli* succinate production. Biotechnol. Biofuels 9, 262. https://doi.org/10.1186/s13068-016-0675-y

Tauxe, R.V., 2002. Emerging foodborne pathogens. Int. J. Food Microbiol. 78, 31–41.

Tran, L., van Baarsel, J.A., Washburn, R.S., Gottesman, M.E., Miller, J.H., 2011. Single-gene deletion mutants of *Escherichia coli* with altered sensitivity to bicyclomycin, an inhibitor of transcription termination factor Rho▽. J. Bacteriol. 193, 2229–2235. https://doi.org/10.1128/JB.01463-10

Ugorakova, J., Bukovska, G., 2003. Lysins and holins: tools of phage-induced lysis. Biologia (Bratisl.) 58, 327–334.

Ushijima, H., Fujimoto, T., Müller, W.E., Hayakawa, S., 2014. Norovirus and Foodborne Disease: A Review. Food Saf. 2, 37–54. https://doi.org/10.14252/foodsafetyfscj.2014027

Voter, A.F., Killoran, M.P., Ananiev, G.E., Wildman, S.A., Hoffmann, F.M., Keck, J.L., 2018. A high-throughput screening strategy to identify inhibitors of SSB protein-protein interactions in an academic screening facility. SLAS Discov. Adv. Life Sci. R D 23, 94–101. https://doi.org/10.1177/2472555217712001

Wang, L., Qu, K., Li, X., Cao, Z., Wang, X., Li, Z., Song, Y., Xu, Y., 2017. Use of Bacteriophages to control *Escherichia coli* O157:H7 in domestic ruminants, meat products, and fruits and vegetables. Foodborne Pathog. Dis. 14, 483–493. https://doi.org/10.1089/fpd.2016.2266

Wojdyla, J.A., Cutts, E., Kaminska, R., Papadakos, G., Hopper, J.T.S., Stansfeld, P.J., Staunton, D., Robinson, C.V., Kleanthous, C., 2015. Structure and function of the *Escherichia coli* Tol-Pal stator protein TolR. J. Biol. Chem. 290, 26675–26687. https://doi.org/10.1074/jbc.M115.671586

Yoshimura, K., Shigeoka, S., 2015. Versatile physiological functions of the Nudix hydrolase family in *Arabidopsis*. Biosci. Biotechnol. Biochem. 79, 354–366. https://doi.org/10.1080/09168451.2014.987207

Yu, L., Wang, S., Guo, Z., Liu, H., Sun, D., Yan, G., Hu, D., Du, C., Feng, X., Han, W., Gu, J., Sun, C., Lei, L., 2017. A guard-killer phage cocktail effectively lyses the host and inhibits the development of phage-resistant strains of *Escherichia coli*. Appl. Microbiol. Biotechnol. 102, 971–983. https://doi.org/10.1007/s00253-017-8591-z

Zborowsky, S., Lindell, D., 2019. Resistance in marine cyanobacteria differs against specialist and generalist cyanophages. Proc. Natl. Acad. Sci. 116, 16899–16908. https://doi.org/10.1073/pnas.1906897116

Zhou, H., Xu, M., Guo, W., Yao, Z., Du, X., Chen, L., Sun, Y., Shi, S., Cao, J., Zhou, T., 2022. The antibacterial activity of kaempferol combined with colistin against colistin-resistant gram-negative bacteria. Microbiol. Spectr. 10, e0226522. https://doi.org/10.1128/spectrum.02265-22