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Effects of Manuka Honey (Methylglyoxal) and Zinc on T4r Bacteriophages

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Abstract

This study examines the effects of two antimicrobic agents (zinc and methylglyoxal) on bacteriophages. Recent studies have suggested Manuka honey has anti-bacterial and anti-viral properties. The main ingredient in Manuka honey is methylglyoxal. Zinc lozenges are marketed as a cold remedy and studies have suggested zinc may shorten the duration of cold signs and symptoms. In order to examine the effectiveness of these chemicals, an assay was developed using the T4r bacteriophage that infects *Escherichia coli*. Bacteriophages follow a similar life cycle as do animal viruses but provide a safe alternative because they do not cause human disease. They also do not require specialized biocontainment facilities or media preparation and can be used in a basic college laboratory. The bacteriophage (T4r) was treated with either methylglyoxal or zinc (in the form of zinc chloride or zinc sulfate) at various concentrations for 10 min. An overnight culture of *Escherichia coli* B was then added, and the mixture was subsequently plated on Luria-Bertani (LB) Agar. The plates were then incubated at 37°C for 48-72 hr to visualize plaque development. Methylglyoxal inhibited plaque formation in a dose dependent fashion from 0.01%-1%. Both zinc chloride and zinc sulfate inhibited plaque formation at concentrations between 50-500 mM and 10-100 mM, respectively. Future studies will examine other antimicrobic agents as well as determine the effects these agents have on the bacteriophages.

Keywords: bacteriophage, methylglyoxal, zinc

1. Introduction

According to the World Health Organization website, three infectious diseases are listed in the top 10 global causes of death, lower respiratory infections, diarrheal diseases and tuberculosis[1]. Antimicrobial agents help reduce the burden of infectious disease however in recent years, drug resistant bacterial strains have arisen. In the US alone, 2 million people are infected with antibiotic-resistant bacteria. Of these, 23,000 die[2]. Drug resistance has also been observed in viruses[3]. Alternative strategies to combat infectious disease therefore are becoming increasingly important.

Bacterial viruses or bacteriophages infect bacterial cells by similar mechanisms observed with animal viruses. These steps include: 1) attachment, 2) penetration, 3) uncoating, 4) steps in nucleic acid and protein synthesis, 5) assembly of viral particle, and 6) release of infectious viral particle. Bacteriophages infect bacterial cells by attaching to specific proteins on the bacterial surface. They enter the bacteria by injecting their DNA into the cell. The virus then takes over the bacterial cell and begins producing viral DNA and proteins. These proteins assemble into a capsid protein that surrounds the genetic material. Finally, the infectious viral particles are released from the cell by lysing (rupturing) the cell. One infected bacterial cell will release 100-150 viral particles. The infection of a bacterial cell can be detected by the formation of a hole or plaque on a lawn of bacterial cells. Recently many labs have suggested the use of bacteriophages as a method for testing antimicrobic chemicals. Bacteriophages have been used as a surrogate for the norovirus, a virus responsible for the stomach flu[4], the adenovirus responsible for respiratory infections[5], and the Ebola virus[6]. Bacterial viruses provide a safe alternative because they do not cause human disease

and therefore do not require specialized biocontainment facilities or media preparation and can be used in a basic college laboratory. Bacteriophages are specific for a given type of bacteria. Many bacteriophages, such as T4, M13 and MS2 infect *Escherichia coli* and their life cycle has been well characterized.

Plant extracts have traditionally been considered to have natural anti-bacterial activity. Researchers have found garlic extract to be effective against *Streptococcus mutans*[7]. Crude extracts of dandelion root demonstrated anti-microbic activity against *Staphylococcus aureus*, methicillin-resistant *S. aureus* and *Bacillus cereus* strains[8]. Dandelion extract successfully inhibited Influenza Type A infections in Madin-Darby canine kidney (MDCK) cells and human lung adenocarcinoma (A549) cells[9].

Similarly, Manuka honey (methylglyoxal) has been studied for its anti-bacterial and anti-viral properties. Recent work in this lab has shown that methylglyoxal inhibits both *Escherichia coli* and *Staphylococcus epidermidis* at concentrations of 5%[10]. Other researchers have found Manuka honey to be effective at eradicating and preventing biofilm formation of *S. aureus*[11]. Manuka honey also demonstrated anti-viral activity against the Varicella-Zoster Virus, the virus responsible for chicken pox and shingles[12] and influenza virus replication[13].

Zinc has also been suggested as an anti-viral agent and has been marketed as a cold remedy. Researchers have shown that zinc lozenges significantly shortened the duration of multiple cold symptoms including nasal discharge, sneezing, and cough[14]. Zinc has been shown to play a role in modulating the binding of proteins to the DNA molecule. DNA primase activity in the T7 phage[15] is modulated by zinc as is the recombinase activity of ϕC31 integrase[16]. In both of these instances, the binding of the protein is mediated by a zinc finger protein. By interfering with these ionic interactions, the metals may affect either viral replication or attachment.

Previous work in this lab developed an assay using the ϕX174 bacteriophage to test various chemicals to determine their anti-viral activities. Using this assay, citric acid[17] and dandelion extract have been shown to decrease plaque formation while garlic extract did not[18]. This study examines the hypothesis that both methylglyoxal and zinc will have anti-viral potential in a bacteriophage T4r assay.

2. Methodology

2.1 T4r Phage Assay

Escherichia coli B was grown in Luria-Bertani (LB) Agar broth overnight at 37°C in a shaking water bath. Diluted bacteriophage T4r was treated with chemicals (methylglyoxal, $ZnSO_4$ and $ZnCl_2$) at the indicated concentrations for 10 min at room temperature. An overnight culture of *E. coli* B was then added to the phage-chemical mixture. This phage-bacteria mixture was added to molten LB soft agar and immediately plated on LB Agar petri dishes. The plates were incubated at 37°C for 48-72 hr to visualize plaque development. Phage titers used in these experiments were approximately 6×10^7 plaque forming units (pfu). Additional controls included plates incubated without phage or with methylglyoxal and zinc alone (no phage). No plaques were observed in either of these conditions.

2.2 Materials

Media, bacteriophage and bacteria were purchased from Carolina Biological (Burlington, NC). Chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Zinc sulfate and zinc chloride were dissolved in water and all chemicals were sterilized using a 0.2 μ filter

3. Results

3.1 Anti-Viral Effects Of Methylglyoxal

Methylglyoxal (MGO), the active ingredient in Manuka honey was tested to determine its anti-viral activity using the T4r phage. Figure 1 shows that MGO inhibits plaque formation at all concentrations tested (1.4 – 69.3 mM). Plaque formation was completely inhibited at concentrations of 69 mM and was half maximal at approximately 1.4 mM. Figure 2 shows the plaques observed in the control (untreated plate) and the inhibition of those plaques on the plate treated with 13.8 mM MGO.

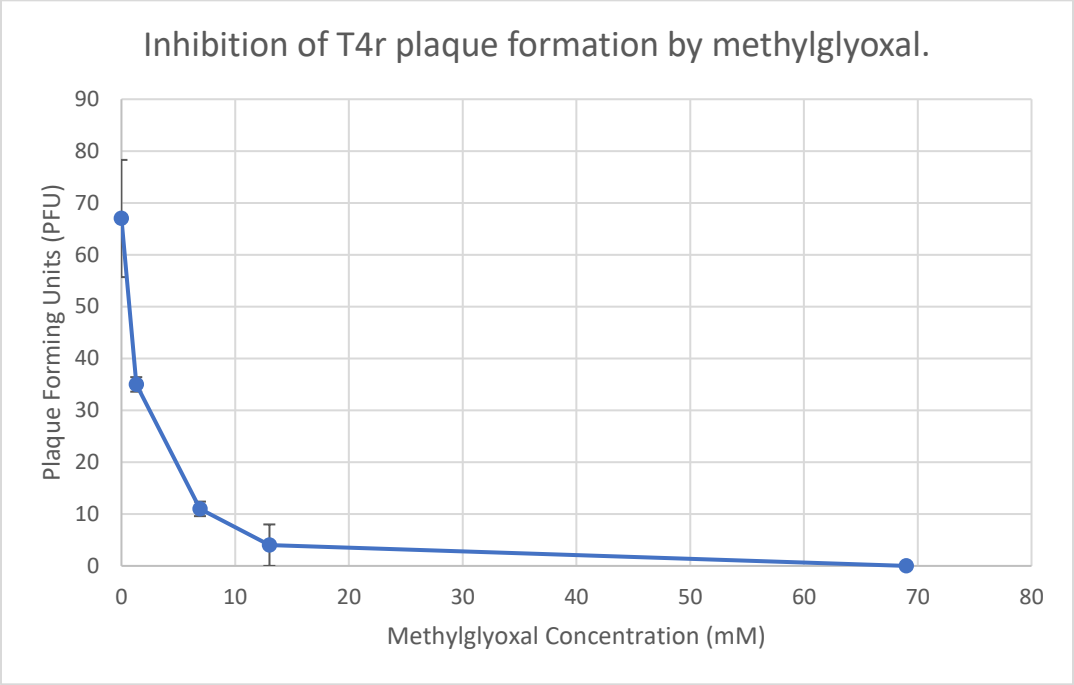

Figure 1. Inhibition of T4r plaque formation by methylglyoxal. Methylglyoxal was incubated with T4r for 10 min. An overnight culture of *Escherichia coli* was added to the phage-chemical solution. The mixture was then added to a tube of soft LB Agar and plated immediately onto LB Agar plates. Following incubation at 37°C of 48-72 hrs, plaque formation was recorded.

Figure 2. LB Agar plates of Bacteriophage T4r. Plate on right was treated with 0.1% methylglyoxal. Plate on the left is untreated.

3.2 Anti-Viral Effects Of Zinc Chloride And Zinc Sulfate

Zinc chloride and zinc sulfate were also tested in the plaque assay (Table 1). Both inhibited plaque formation in a dose dependent fashion. Inhibition of plaque formation was observed at 10 mM for zinc sulfate and plaque formation was completely inhibited at 250 mM. Zinc chloride was inhibitory at 250 mM and showed almost complete inhibition at 500 mM.

Table 1. Comparison of Zinc Sulfate and Zinc Chloride on Plaque Formation

Concentration (mM)	$ZnSO_4$ (pfu, % controls)	$ZnCl_2$ (pfu, % controls)
0 mM	100	100
10 mM	67	99
50 mM	35	86
100 mM	48	99
250 mM	0	39
500 mM	0	2

Solutions of $ZnSO_4$ or $ZnCl_2$ were incubated with T4r for 10 min. Following the addition of an overnight culture of *E. coli* B, the mixture was added to a tube of molten LB soft Agar and plated immediately onto LB Agar plates. Plaque formation was observed following incubation at 37°C for 72 hrs.

4. Discussion

Bacterial viruses which infect *E. coli,* such as T4r and ϕX174, provide a safe alternative for testing anti-viral chemicals in the college laboratory. Bacterial viruses do not cause human disease and therefore do not require specialized biocontainment facilities or media preparation and are well-suited for use in a basic college laboratory. This study demonstrated that both methylglyoxal, and zinc exhibited anti-viral effects on T4r bacteriophage by inhibiting plaque formation. This inhibition of plaque formation was dose dependent suggesting that MGO and zinc have anti-viral properties and may prove useful in the treatment of viral diseases. For example, MGO is marketed in bandages and may be useful for the treatment of blisters caused by the Varicella Zoster Virus. Zinc lozenges are already marketed in shortening the duration of the common cold.

Methylglyoxal is a potent protein glycating agent. The dicarbonyls of MGO attack amino groups associated with lys and arg residues and can form advanced glycation end products[19]. The Safety Data Sheet (SDS) for MGO available from Sigma-Aldrich indicates no toxicity data is available[20]. However, the SDS found for a similar methylglyoxal compound listed an LD50 of 1165 mg/kg in rats[21]. The concentrations of MGO used in this study range from 10 μg to 500 μg and are lower than that listed. While MGO has been identified as the Unique Manuka Factor with anti-bacterial activity in Manuka Honey, the Unique Manuka Factor varied over a sampling of Manuka honey purchased over the counter[22] and it is therefore difficult to correlate our MGO concentrations with that found in Manuka honey.

Manuka honey has been suggested to aid in wound healing[23] and is used in bandages available over the counter for topical treatment. Another study, however, has suggested that the use of MGO may delay wound healing in diabetic patients due to the increase in advanced glycation end products[24]. Several studies have examined the effect of MGO on erythrocytes and leukocytes. Concentrations of 5 mM MGO resulted in increased osmotic fragility of erythrocytes and 10 mM resulted in increased hemolysis[25]. Concentrations of 5 and 10 mM MGO affected agranulocyte viability while granulocyte viability was not significantly affected[25]. Concentrations of 400 μM MGO accelerated the aging process in human skin fibroblasts in culture[26]. While deleterious *in vitro* effects of MGO have been documented, *in vivo* studies with MGO are limited[27]. Therefore more work is required to help weigh the potential benefits of MGO with the deleterious effects observed.

In comparing the results observed with $ZnSO_4$ and $ZnCl_2$, it is interesting to note that the inhibition of plaque formation was greater at lower concentrations for $ZnSO_4$ than for $ZnCl_2$. Zinc has been suggested as an anti-viral prevention for the common cold[14] and is available over the counter as lozenges to reduce the duration of a cold. This study does show a reduction in the number of plaques produced at higher concentrations of zinc.

This study shows Methylglyoxal and zinc do have anti-viral properties. Future work on this project will begin to examine the effects of other anti-viral chemicals including triclosan and thimerosal as well as examining the effects

of these anti-viral agents on other bacteriophages. For example, while T4r attaches to the diglucosyl residues in the lipopolysaccharide of *E. coli* B and injects the phage DNA into the host cell[28], the phi6 bacteriophage fuses with the outer membrane of the Gram-negative *Pseudomonas* species to enter the cell[29]. These differences may be helpful in examining the mechanism by which these anti-viral agents inhibit plaque formation.

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