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RESEARCH ARTICLE

Controlling foodborne pathogenic *Citrobacter freundii* via lytic bacteriophages

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Abstract

Recently, foodborne diseases caused by pathogenic bacteria have been expanding. This requires searching for ways to control or get rid of these diseases. Using bacteriophages to control, prevent and treatment of foodborne diseases is one of the important actions due to their killer effect on bacteria. Many species of pathogenic bacteria can be transmitted through food, including *Citrobacter freundii* which can cause food poisoning, urethritis, abscess, and meningitis in infants. This study aimed to isolate and characterize bacteriophages specific to *Citrobacter freundii* to be used to control the contamination of food with *Citrobacter freundii*. *C. freundii* and its specific bacteriophages were isolated from sewage water. Isolated phages were found to be belonging to two phage species. These two phages had a head and tail and were designated Citro 1 and Citro 2. Phage Citro 1 has a long contractile tail and Citro 2 has a long non-contractile tail. Therefore, they were classified into Family *Myoviridae* and Family *Siphoviridae*, respectively. Application of bacteriophages to green salad artificially contaminated with *C. freundii* resulted in a high reduction in the density of *C. freundii* in green salad kept at both room temperature and at 4°C. Moreover, when the density of *C. freundii* decreased the number of phage particles increased. Such results may indicate that bacteriophages can be used to control the contamination of foodstuffs with pathogenic bacteria.

Keywords: Bacteriophages; *Citrobacter freundii;* Foodborne diseases; Pathogenic

Introduction

The genus *Citrobacter* belongs to *Enterobacteriaceae* and consists of about 13 species, and among these, *Citrobacter freundii* which can infect humans causing foodborne disease. *Citrobacter freundii* are found in water, soil, and the human or animal intestines. (Borenshtein and Schauer, 2006). Previously, these bacteria had low virulence, but recently shifted to pathogenic bacteria (Liu *et al.*, 2018), Some studies conducted in North America have stated that the *Citrobacter* genus is responsible for 3 to 6% of *Enterobacteriaceae* infections (Borenshtein and Schauer, 2006). The treatment of infections with some C*itrobacter* strains has become difficult and complicated due to acquired resistance to many antibiotics as β-lactam antibiotics (Porres-Osante *et al*., 2015). The resistance to antibiotics acquired by many species of bacteria including *Salmonella*, Staphylococcus, and *Citrobacter* is due to extensive and unjustified use of antibiotics, where appeared multidrug-resistant mutants. Therefore, efforts should be made to find different agents to control food contamination with some pathogenic bacteria (e.g. *E. coli* and *Citrobacter*). Where the lytic bacteriophages were successfully used as antibacterial agents due to their ability to infect bacterial cells and

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kill host cells, releasing many new phages that can infect other cells. The objective of this study was the isolation and characterization of bacteriophages specific to the most common bacterial contaminant (*e.g. Citrobacter*). Moreover, efforts will be made to use the isolated phages to control food contamination with this bacterium.

Materials and methods

The used bacteria

Citrobacter freundii was isolated from a sewage water sample and identified by the VITEK2 system in the Microbiological Lab. of Sohag University Hospital.

Isolation of bacteriophages

Isolation of bacteriophages sewage water samples were used to isolate bacteriophages which specific to *C. freundii*. The liquid enrichment technique of Adams (1966) with minor modification as described by Hammad (1989) was used to isolate phages. Nutrient broth medium (Allen, 1959) was used for liquid enrichment.

Phage detection

The spot test on nutrient agar double-layer plates was used to detect bacteriophage as described by (Borrego *et al*., 1987).

Purification of bacteriophage

The technique of single plaque isolation was used to collect pure single isolates of phages according to Borrego *et al*., (1987).

Preparation of high titer

The agar double-layer method was used for preparing the high titer of bacteriophage suspension for each one as described by Maniatis *et al*. (1982).

Titer estimation

The titer of bacteriophage was estimated by using the method described by Kiraly *et al*. (1970).

Characterization of the isolated phages

The optimum pH for infection

Sterile plastic Wasserman tubes containing 1 ml SM buffer with different pHs (*i.e.* 3.0, 4.0, 5.0, 6.0 up to 12.0) were prepared. The pH was regulated with NaOH (1M) and HCl (1M). Every single isolate of bacteriophages was transferred to the prepared tubes (plaque/tube). then, incubated at 30°C for 60 min. then 5µl from each one was spotted over double-layer agar plates (four replicates), containing the appropriate indicator bacterium (*C. freundii***)**. The diameter of the lysed zone was measured and then were calculated the average values of the replicates.

Stability to UV radiation

Petri dishes containing 5 ml of high titer bacteriophage suspension of each isolate were placed 20 cm away from the germicidal UV lamp with wavelength 260 nm. after 5, 10, 20, 30, and up to 80 min. ten µl of each irradiated bacteriophage suspension were spotted over double-layer agar plates, which contained the indicator bacterium (*C. freundii*). Plates were examined for lysed zones after 24 h incubation at 30-33°C.

Thermal inactivation point

The sterile glass wasserman tubes each containing 1 ml of high titer bacteriophage suspension of each isolate. Tubes were heated in the water bath for 10 min. on different thermal degree (65, 70, 75, 80, … up to 100°C), then were cooled under tap water. After heat treatment 10µl from each tube was spotted over double-layer agar plates containing the indicator bacterium (*C. freundii*). Plates were examined for lysed zones after 24h incubation at 30-33°C.

Electron microscopy

The electron microscope grids formvar 200 mesh coated were prepared to all isolated bacteriophages (grid/isolte), then were stained with 0.5% uranyl acetate pH 4.5 for 15 - 30 seconds (Stacy *et al*., 1984). all grids were examined by transmission electron microscope at 50 kv in (JEM 100 CXII) in Assiut University, Assiut, Egypt.

Controlling contamination of green salad with C. freundii via application of lytic bacteriophages

Samples of vegetables including cucumber, tomato, carrots, and green pepper were washed with sterilized water and disinfected with ethanol 70%. Vegetables were cut approximately 10 mm in thickness and 10 mm in diameter, and the salads were placed in sterilized plastic plates (100 gm/each). Plates were divided into groups and subjected to the following treatments:

1- Mixed with 500µl of liquid bacterial culture (10^8 cfu/ml) of *C. freundii*.

2- Mixed with 500µl of liquid bacterial culture of *C. freundii* plus 2 ml of specific bacteriophage suspension with titer (10^9 pfu/ml).

3- Untreated sample as a control, the total count of *C. freundii* and the specific bacteriophages for all treatments were assayed at zero time and after storage for 24 hrs. at 4ºC and at room temperature, two replicates were involved for each treatment.

Results and Discussion

The bacterial isolate

Bacterial isolates were obtained from sewage water samples. The light microscopic examination indicated that this bacterial isolate is Gram-negative and has short rods in shape. The isolated bacterium was identified to be *Citrobacter freundii* by the VITEK2 system in the Microbiological Lab. of Sohag University Hospital. Lipsky *et al.* (1980) stated that *C. freundii* opportunistic pathogenic bacteria can cause many infections including urinary tract infections, respiratory, wounds, and bloodstream (bacteremia). Moreover, drug-resistant strains have also been observed especially in healthcare settings.

Bacteriophages

Lytic bacteriophages were isolated from samples of sewage water the spot test indicated that bacteriophages of *C. freundii* were commonly found in the sewage water samples. As shown in Figure (1) Similarly, Mizuno *et al.* (2020) isolated bacteriophages specific to *C. freundii* from water treatment plants.

Purification of bacteriophages

The criteria used to differentiate bacteriophages specific to various bacteria include Plaque morphology and diameters, where the shape, size, and outline of the plaques are characteristic of the phage strain (Hammad, 1989), single plaques were used to pick up pure bacteriophage isolates, figure (2) shown the bacteriophages specific to *C. freundii* formed different morphologies of single plaques, clear in appearance and circular of 1 to 3 mm in diameter. different morphological plaques were selected (five plaques) and kept as individuals.

The high titer of bacteriophage

Fifty ml of bacteriophage suspension was prepared for each isolate from five phage isolates. The titers of every one of them ranged from $2.7x10^{10}$ to $4.5x10^{10}$ pfu/ml.

Characteristics of the isolated phages

The optimum pH for phage infection

The ability of bacteriophage isolates to infect *C. freundii* was studied at different pH values (pH 3-12). As shown in Table (1), lysed zones were formed by all five phage isolates at all pH levels (pH 3-12). Similarly, many previous studies refer to the stability of phages to different pH values, were reported by Roslycky *et al.* (1962); Challaghan *et al.* (1969); Hammad and Ali (1999); Fathy (2008). Whereas, bacteriophage isolates No. 1 and 4 have optimum infectivity (formed widest spots) at pH 8. Moreover, bacteriophage Isolates No. 2, 3, and 5 have optimum infectivity (formed widest spots) at pH 7. These results may indicate that bacteriophages No. 1 and 4 are similar in their optimum pH for infection. Therefore, two isolates of phages (No. 1 and 4) are named (Group A). while isolates No. 2, 3, and 5 are named (Group B) since they are similar in their optimum pH for infection (pH 7).

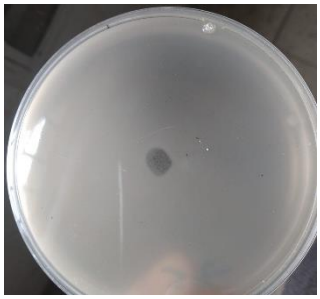

Figure 1. The lysed zone seen after incubate spotted with a drop of the prepared phage lysate

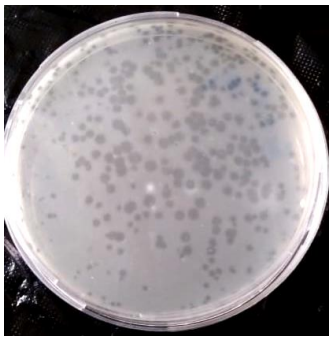

Figure 2. shows the differences in plaque morphology of bacteriophages specific to *C. freundii.*

Table 1. Stability of phages specific to *C. freundii* to different pH values.

Phage groups	Phage isolate No.	pH levels									
		3	4	5	6	7	8	9	10	11	12
A	1	10.1	11.0	12.4	12.9	13.2	15.7	14.0	12.3	11.0	9.8
	4	9.9	11.3	11.8	12.0	12.9	14.8	12.2	11.7	9.9	8.3
	2	9.7	10.5	11.3	12.9	14.1	12.3	11.7	9.0	7.9	6.3
B	3	8.9	9.2	10.3	11.8	13.8	12.3	11.4	10.0	8.7	6.4
	5	9.9	10.8	11.4	12.1	14.2	12.0	11.2	8.9	7.6	5.1

Stability of phages to Ultraviolet radiation

260 nm wavelength UV radiations inactivated the isolated phages at different exposure times (Table 2). Accordingly, the bacteriophages specific to *C. freundii* under study were divided into two groups. Each group comprised bacteriophage isolates which inactivated after the same exposure time. Group (A) contained phages (No. 1 and 4) which were inactivated after UV exposure for 30 min. while, Group (B) comprised phages No. 2, 3, and 5 since they inactivated after UV exposure for 40 min. Interestingly, the phages of *C. freundii*, which were divided into groups (A and B) based on the optimum pH, were found to have the same stability as UV radiation. These results may indicate that the bacteriophages of each group represent a single phage type. Elsharouny (2007) established that phage isolates of either *Azotobacter* or *Azospirillum*, which belong to one phage type, have the same stability to radiation of UV.

Table 2. Effect of UV radiation (260 nm) on *C. freundii* bacteriophages

Phage groups	Phage isolate No.	Exposure time							
		10	20	30	40	50	60	70	80
A	1	+	+	-	-	-	-	-	-
	4	+	+	-	-	-	-	-	-
	2	+	+	+	-	-	-	-	-
B	3	+	+	+	-	-	-	-	-
	5	+	+	+	-	-	-	-	-

Thermal inactivation point

The thermal inactivation point is used by the bacteriophages as a characteristic of isolates. Hammad (1993) and Hammad and Ali (1999) refer to that the different bacteriophage types of *B. japonicum* displayed different thermal inactivation points. Data presented in Table (3) indicated that phage isolates of *C. freundii* No. 1 and 4 were inactivated after 10 min at 85°C, while phages No. 2, 3, and 5 lost their ability to infect bacteria at 100°C after 10 min. These results may indicate that since, phages No. 1 and 4 which were included in group (A) exhibited the same optimum pH, sensitivity to UV, and thermal inactivation point it is likely for these two phages (1 and 4) to belong to one phage type. While, phages No. 2, 3, and 5 in group (B) have the same optimum pH, sensitivity to UV, and thermal inactivation point. Therefore, these three isolates may belong to another phage type.

Table 3. Thermal stability of *C. freundii* bacteriophages, exposed to 65 –100°C for 10 min

Phage groups	Phage isolate No.	Temperature (°C)							
		65	70	75	80	85	90	95	100
A	1	+	+	+	+	-	-	-	-
	4	+	+	+	+	-	-	-	-
	2	+	+	+	+	+	+	+	-
B	3	+	+	+	+	+	+	+	-
	5	+	+	+	+	+	+	+	-

Size and morphology

Bacteriophage isolates of each group (groups A and B) were stained by negative stain and then examined by electron microscope. All isolates were found to have head and tail. According to the International Committee on Taxonomy of Viruses (ICTV), these five phage types belong to Order *Caudovirales*. As shown in Table (4), based on the bacteriophage particle dimensions, the isolates of each group were found to be similar in length and width of their tails as well as in their head diameters. Generally, phages of each group exhibited similar particle dimensions. Accordingly, the isolates of each group may represent one bacteriophage type. *i.e.* no doubt, the five phage isolates belong to two types. Phages No. 1 and 4 of Group (A) were found to be one phage type of long contractile tail (Figure 3) belonging to Family *Myoviridae* and designated ∅

Citro 1. The three phage isolates No. 2, 3, and 5 of group (B) were similar in their particle dimensions and morphology. Therefore, these three phage isolates belong to one phage type and are designated ∅ Citro 2. This phage has a long non-contractile tail and it could be classified under Family *Siphoviridae*. Francki (1973) referred to there are several different factors that can affect phage particle size during preparative procedures. Therefore, it is making difficult to valid comparisons between published micrographic data. Accordingly, there may be a group of phages that belong to the same type but differ in micrograph characteristics, despite that, these phages of each group showed the same thermal inactivation point, the same sensitivity to UV radiation, and the same optimum pH, which confirm that these five phage isolates belong to two phage types.

Controlling contamination of green salad with C. freundii pathogen via lytic bacteriophages

Several studies have been published on the use of phages for controlling *Salmonella* and *Citrobacter* in vegetables. Leverentz, *et al.* (2001) reported that a mixture of four phages markedly reduced the number of *Salmonella enteritidis* on melon slices at 5ºC and 20ºC. Moreover, Mizuno *et al.* (2020) isolated and characterized lytic phages of *Citrobacter rodentium* and found that the isolated phages reduced the number of *Citrobacter rodentium* in liquid culture. They stated that these bacteriophages can be used in the future as phage-therapy. In this study, the isolated phages of *C. freundii* were used as antimicrobial agents to control the contamination of green salad with *C. freundii.* As shown in Tables (5 and 6) the number of *C. freundii* markedly increased in green salad treated with the liquid culture of *C. freundii* from 11.5 $x10^5$ cfu/g. at zero time to 570 $x10^5$ cfu/g. and 215.2 $x10^5$ cfu/g. after 24 hrs at room temperature and at 4ºC, respectively. On the other hand, in the presence of bacteriophages number of *C. freundii* reduced from 11.5 $x10^5$ cfu/g. at zero time to 3.6 $x10^3$ cfu/g. and 2.21 $x10^3$ cfu/g. after 24 hrs. With decreasing the density of *C. freundii* in green salad due to the application of phages number of phages increased from 40.9 x 10^9 pfu/g. at zero time to 90.8 x 10^9 pfu/g. after 24 hrs at room temperature. Moreover, at 4ºC reduction in density of *C. freundii* was recorded due to the application of bacteriophages. The recorded number of *C. freundii* was 2.10 $x10^3$ cfu/g. after 24 hrs and high number of phages was recorded (78.8 x 10^9 pfu/g.). Such results may indicate that bacteriophages specific to *C. freundii* could be used as antimicrobial agents to get rid of these pathogenic bacteria (*C. freundii*) or at least reduce their density in contaminated vegetables. Hassan and Khalaphallah (2023) found that *Aspergillus terreus* isolated from healthy rhizosphere soil around healthy bean roots could suppress *Fusarium oxysporum* the causal agent of bean root rot in Upper-Egypt *in vitro* experiment.

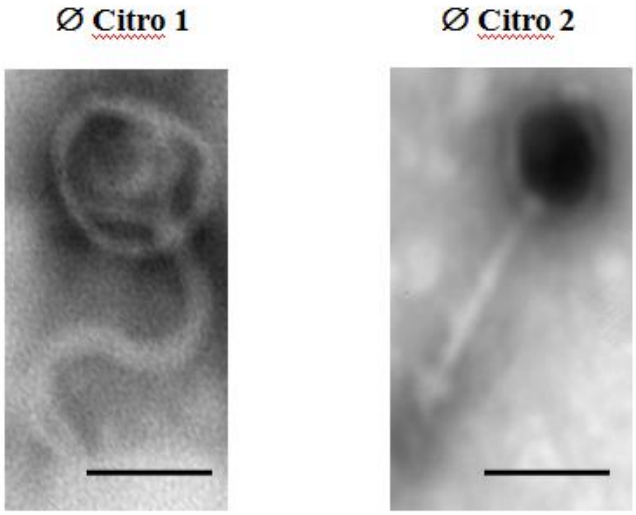

Figure (3): Electron micrographs of negatively stained phage particles specific to *Citrobacter freundii.* Magnification bar = 100 nm

Table 4. Measurements of bacteriophage particles specific to *C. freundii*

Phage groups	Phage isolate No.	Head diameter ± SD (nm)	Tail Length ± SD (nm)	Tail Width ± SD (nm)
A	1	127 ± 2	219 ± 3	22 ± 2
	4	125 ± 3	221 ± 4	20 ± 2
B	2	89 ± 3	194 ± 2	9± 3
	3	92 ± 2	196 ± 3	10 ± 2
	5	88 ± 3	194 ± 3	11 ± 2

SD = Standard deviation

Table 5. Effect of bacteriophages on the density of *C. freundii* in green salad kept at room temperature for 24 hrs.

Sampling Time	The used Bacteria	Treatments: Count of bacteria (10^5 cfu/gm) Untreated	Count of bacteria (10^5 cfu/gm) Treated With bacteria	Treated with bacteria and bacteriophage: Count of bacteria (10^3cfu/g)	Treated with bacteria and bacteriophage: Count of Bacteriophage (10^9pfu/g)
Zero time	*Citrobacter freundii*	0.0031	11.5	3.6	40.9
After 24 hrs.	*Citrobacter freundii*	9.8	570	2.21	90.8

Table 6. Effect of bacteriophages on the density of *C. freundii* in green salad kept at 4°C for 24 hrs.

Sampling Time	The used Bacteria	Treatments			
		Count of bacteria (10^5 cfu/gm)		Treated with bacteria and bacteriophage	
		Untreated	Treated With bacteria	Count of bacteria (10^3cfu/g)	Count of Bacteriophage (10^9pfu/g)
Zero time	*Citrobacter freundii*	0.0031	11.5	3.6	40.9
After 24 hrs.	*Citrobacter freundii*	9.7	215.2	2.10	78.8

Conclusion

Isolated phages were discovered to come from two different phage species. Citro 1 and Citro 2 were the names given to these two phages, which had a head and a tail. Citro 1 possesses a long contractile tail, whereas Citro 2 possesses a long non-contractile tail. As a result, they were assigned to the Families Myoviridae and Siphoviridae, respectively. The application of bacteriophages to green salad that had been intentionally contaminated with *C. freundii* resulted in a significant reduction in *C. freundii* density in green salad held at both room temperature and 4°C. Furthermore, when the density of *C. freundii* declined, so did the quantity of phage particles. Such findings may imply that bacteriophages can be employed to control harmful bacteria contamination of crops.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are included in this published study.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

Equal contributions.

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