Journal of Materials and Environmental Science
ISSN : 2028-2508
e-ISSN : 2737-890X
CODEN : JMESCN

J. Mater. Environ. Sci., 2022, Volume 13, Issue 09, Page 1056-1066

http://www.jmaterenvironsci.com

Effect of Temperature on Bacteriophage-mediated Lysis Efficiency with a Special Emphasis on Bacterial Temperature History

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Received 24 Aug 2022,
Revised 21 Sept 2022,
Accepted 25 Sept 2022

Keywords
- ✓ *Bacteriophage,*
- ✓ Temperature dependence,
- ✓ Lysis efficiency,
- ✓ Temperature sensitive,
- ✓ Plaque size.

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Abstract
Despite the great potential of phages as biocontrol agents, there is much uncertainty about the environmental factors influencing lysis efficiency. In this study we investigated the effect of temperature using three distinct lytic *E. coli* phages that were isolated from a single environmental water sample. All three were identified as dsDNA phages belonging to the Myoviridae family. Whereas the optimal growth temperature of *E. coli* is well known to be 37 ˚C and exposure of phages (prior to mixing with bacteria) to temperatures between 4 and 37˚C did not affect their infectivity, plaque sizes and numbers greatly decreased with increasing incubation temperature (20˚C, 30˚C, 37˚C) of the phage-host mix. At 37˚C, no visible plaques were observed. Results suggest that temperature sensitivity of the phage-host interaction is distinct from the temperature susceptibility of the two players and corroborate previous reports that highest lysis rates are obtained at temperatures approximate with ambient conditions of the phage environment. Infectivity was however found not only to depend on the incubation temperature of the phage-host mix, but also on the bacterial temperature history. Moreover, exposure of bacteria to heat stress prior to phage challenge resulted in a phage-resistant phenotype raising the question whether bacterial pathogens shed from warm-blooded hosts might be less susceptible to phages adapted to environmental temperature conditions.

1. Introduction

Bacteriophages (herein abbreviated as phages) have the unique ability to eliminate bacteria with great specificity. It is estimated that phages in the environment are 5-10 fold more abundant than bacteria and are thus the most abundant biological entities on Earth [1]. They can be assumed to have a pronounced impact on microbial ecology. Phages have furthermore long been recognized as biotherapeutic agents to combat bacterial infections (for example for treating wounds; [2] and more recently for biological disinfection in food production [3]. Although phage isolation and use seem straightforward, there are many pitfalls to successful application. One of the greatest obstacles is insufficient knowledge of parameters influencing phage lysis efficiency. This study aimed to shed more light on the temperature-dependent nature of phage-host interactions. A number of studies have revealed that temperature can be an important determinant affecting efficiency of bacterial removal. Most studies were linked to dairy research due to the economic damage potentially inflicted by phages on bacterial starter cultures and dairy production lines and the resulting strong interest to identify

conditions where bacterial hosts were less susceptible [4] [5] [6]. Both inhibition of phage-mediated lysis at low temperatures (relative to the optimal growth temperature of the bacterial host; [7] [8] and at high temperatures has been reported [4] [5]. Temperature dependence was later also reported for bacteriophages relevant for control of food pathogens like *Listeria* [9], *Salmonella* and *Campylobacter* [10]. For coliphages [11] differentiated between three physiological types based on the effect of temperature on their 'plaquing' properties and introduced the terms of high temperature (HT; ≥25˚C), mid-temperature (MT; 15-42˚C) and low temperature (LT; ≤30˚C) phages. Temperature profiles of bacteriophages from different habitats showed that phages isolated from faeces or sewage tended to belong to the LT or MT type, whereas phages isolated from a river tended to belong to the LT type. Plaque numbers of LT phages dropped sharply to zero at temperatures between 33 and 37˚C. More recently [12], suggested in this context that phages are 'locally adapted' to their bacterial hosts. Whereas [11] reported that the 'maximum and minimum plating temperatures to be stable properties of the phages and that were not influenced by the growth temperature of the host', [9] assigned the temperature-dependent susceptibility to the bacterial host. An epidemic *Listeria monocytogenes* strain was identified that was able to avoid phage-mediated lysis by broad-host-range phages when bacteria were grown at 37˚C, but not when grown at 30˚C. Resistance was regardless of the temperature during infection or subsequent incubation of bacteria-phage mixtures. As different phages can respond in different ways to changing environmental parameters, it is essential to select the right conditions when isolating and applying environmental phages. The choice of the correct temperature is particularly important in the case of human pathogens where the optimal growth temperature (typically the one encountered in the human host) is very different from the temperatures prevalent in the environmental lifecycle of those pathogens. Whereas phage screening at the optimal bacterial growth temperature makes sense for therapeutic applications, suboptimal results might be obtained for phage-based biological disinfection that is typically performed at ambient temperature. This study was performed with three distinct coliphages that were isolated from a single brook water sample and that were chosen on the basis of different plaque sizes. With all three of these exemplar environmental phages, a strong temperature impact on lysis activity was observed after mixing them with their bacterial host. We aimed to examine whether it is the temperature susceptibility of the phages or the bacterial host causing temperature sensitivity of the host-phage mixture and to which extent phage lysis activity depends on the temperature history of phages and bacteria.

2. Methodology

2.1 Bacteria and growth conditions

The study was performed with *Escherichia coli* ATCC 25922, which is a clinical isolate. Bacteria were grown overnight on tryptone soya agar (TSA; CM0131; Oxoid Ltd Basingstoke, Hampshire, UK) at 30ºC. Liquid cultures were obtained by inoculating 10 mL of tryptone soya broth (TSB; CM1016; Oxoid Ltd Basingstoke, Hampshire, UK) in 50 mL Falcon tubes followed by overnight incubation at 25ºC (or indicated temperature) at a 45º angle at 250 rpm in a temperature-controlled mini shaker (cat. number 980151, VWR, USA). TSB broth and TSA plates were routinely amended with 5 mM $CaCl_2$ (added as $CaCl_2$*$7H_2O$) to allow for efficient phage adsorption. Cell density was measured in a spectrophotometer (JENWAY 6310, Camlab, England, UK) at 600 nm (OD_{600}) and adjusted to an OD_{600} = 1.0 by dilution with TSB equilibrated to room temperature. For experiments addressing temperature-dependent phage resistance, aliquots of 10 mL of overnight cultures and optical density-adjusted cultures were harvested by centrifugation (5,000 g, 5 min). Resulting cell pellets were re-suspended in phosphate buffered saline (PBS) and exposed to treatment temperatures for 24 hours

using standard laboratory heat blocks or incubators. Cell aliquots were subsequently equilibrated to room temperature, harvested by centrifugation and re-suspended in TSB. For experiments addressing effect of elongated temperature exposure on phage infectivity, 1 mL of phage stock (10^9 PFU/mL) was exposed to the indicated temperature in microcentrifuge tubes using standard heat blocks or incubators.

2.1 Sampling, enrichment and isolation of bacteriophages

Water samples were collected from Chicheley brook flowing across the Cranfield University campus at a location where the brook water mixes with treated effluent discharge from the Cranfield University wastewater treatment plant. The water temperature of the brook does not exceed 20˚C even in hot summer months and when there are low water levels. Samples were passed through a 0.22 µm membrane filter (Millex GP, Merck Millipore, Darmstadt, Germany) to remove bacteria and the filtrate containing phages was collected in a sterile 50 mL Falcon tube.

Bacteriophages specific for *E. coli* were subsequently enriched by mixing 10 mL of the filtrate with an equal volume of double strength TSB (supplemented with 5 mM $CaCl_2$) and inoculating the mixture with 0.4 mL of an overnight *E. coli* culture (grown as described previously) followed by overnight incubation (30ºC; 250 rpm). Incubation was performed until clearance indicated bacteriophages-mediated cell lysis (typically after 24 h). Chloroform was subsequently added to a final concentration of 2% to eliminate remaining intact bacteria followed by centrifugation at 5,000 *g* for 5 min. The supernatant was filtered using 0.22 µm membrane filter (Millex GP, Merck Millipore, Darmstadt, Germany). Appropriate dilutions of enriched sample were plated using the soft agar overlay method [13]. Plates were examined for plaques after overnight incubation at 30ºC. Selected plaques were transferred into SM buffer (100 mM NaCl, 8 mM $MgS0_4*7H_20$, 50 mM Tris-Cl, adjusted to pH 7.5), re-suspended and, serially 10-fold diluted for re-isolation.

2.3 Purification of bacteriophages

Bacteriophages were isolated three times using a modified soft agar overlay method [13] (with some modifications) to ensure purity. A mixture of 100 µL of viral concentrate serially 10-fold diluted in SM buffer and 150 µL of log-phase *E. coli* were added to 3 mL of molten 0.7% TSB agar (maintained at 48ºC), mixed immediately by gentle vortexing and then distributed evenly over TSA agar plates supplemented with $CaCl_2$ to a final concentration of 5 mM. The soft agar was allowed to solidify for 20-30 min and plates were incubated overnight at 30ºC (or indicated temperature) to allow for plaque formation. Isolated plaques were picked using sterile wooden toothpicks to inoculate 5 mL log-phase *E. coli* cultures followed by incubation at 30ºC with shaking (250 rpm) for 8 hours. Lysate from single plaques were treated with chloroform to a final concentration of 2%, mixed and centrifuged at 5,000 g for 5 min. The phages were recovered from the upper phase suspension and passed through 0.22 µm filter (Millex GP, Merck Millipore, Darmstadt, Germany). Phage stocks were stored at 4ºC.

2.4 Phage challenge experiments and monitoring of optical densities

Phage challenge experiments were performed in 50 mL conical centrifuge tubes (Fisherbrand, Fisher Scientific UK Ltd., Loughborough) by adding bacteriophage stock solution to 10 mL of bacterial suspension adjusted to an optical density OD_{600} of 1.0. In all experiments 10^9 PFU/mL were applied corresponding to a multiplicity of infection (MOI) of 1.

Temperature stress was performed by exposing six bacterial aliquots of 1 mL each (bacteria re-suspended in PBS, pH 7) to different temperatures (4, 20, 37, 41, 44 or 50°C) for 24 hours. Cells killed at 70°C for 15 min served as control. After stress exposure, cells were harvested by centrifugation (5000 g for 5 min) washed in 1 mL TSB, and re-suspended in 9 mL TSB to obtain a final concentration

of approximately 10^9 cells/ml. Temperature-exposed bacteria were mixed with phage as described earlier and shaken at 250 rpm at 20°C. Accordingly, phages were exposed to different temperatures (4, 20, 37, 44 or 50°C) for 24 hours after dilution in SM buffer to 10^9 pfu mL^{-1}. Phages were subsequently serially diluted in SM buffer in steps of 10-fold prior to spotting 1.5 µL aliquots on square culture plates (Greiner, 688102, Sigma-Aldrich, UK) containing TSA (supplemented with $CaCl_2$ to a final concentration of 5 mM) and with pre-spread *E. coli* (prepared by spreading a mixture of 9 mL 0.7% TSB agar, maintained at 48ºC, and 450 µL of log-phase *E. coli).* Plates were incubated overnight at 20˚C for plaques to develop.

Optical densities of cell suspensions were measured on a TECAN M200 Pro plate reader (Tecan UK Ltd, Reading, UK). Samples of 1 mL were transferred into transparent 48 well tissue culture plates (non-treated, flat bottom, cat. nr. TCP001048; Jet Biofil, Braine I'Alleud,Belgium) and absorbance at 600 nm was measured. Readings were typically taken directly after addition of phages and every hour after the challenge or after 6, 12, 24 and 36 hours (as indicated).

2.5 Transmission electron microscopy

Samples were prepared by pipetting 3lL of phage sus- pension (*109 PFU mL-1) onto negatively glow discharged 10lm thick C-Flat™ carbon grids (400-mesh) followed by a 1min incubation. Sample droplets were then partially blotted on Whatman™ quantitative filter paper, Grade 1 followed by application of 3lL of 2% uranyl acetate stain solution. After 1min of staining, excess fluid was removed from the grid surface by positioning the filter in an angle and air drying the sample for approximately 3–5min. The sample on each grid was then imaged in low-dose conditions on a Tecnai 10 transmission electron microscope (FEI Company, Oregon) operating at 100keV. Images were taken at 20 K magnification and captured by using a Gatan Ultrascan 4000 4 k·4k CCD camera equipped with an ultrasensitive phosphor scintillator (Gatan) to produce a final pixel sampling of 11 Angstroms per pixel.

3. Results and Discussion

Phages were isolated from a brook water sample on the basis of the plaque sizes produced when incubating bacteria-phage mixtures at ambient room temperature (20˚C; Fig. 1A). Distinct phages resulting in big, medium and small plaques were referred to as phage B, M, or S (Fig. 1B).

3.1 Effect of incubation temperature on plaques sizes and numbers

Plaque sizes and numbers were compared when incubating bacteria-phage mixtures (embedded in soft overlay agar) at 20, 30 and 37˚C. For all three phages, greatest plaque sizes were obtained when incubating plates at 20˚C (Fig. 2). Plaque sizes decreased when raising the incubation temperatures to 30˚C and plaques were not visible at all for phages B and M at 37˚C. In agreement with plaque sizes, plaque numbers decreased with increasing incubation temperatures as indicated in Fig. 2.

3.2 Temperature susceptibility of phage

In order to identify whether temperature susceptibility of the bacteriophages or the bacteria was responsible for the observed phenomenon, purified phage suspensions were exposed to different temperatures (4, 20, 30, 37, 44 or 50˚C) for 24 hours. Serial 10-fold dilutions of such treated phage suspensions were subsequently spotted on agar plates with pre-aliquoted *E. coli* and incubated at 20˚C (Fig. 3). For phage B, plaques generated by diluted phages were comparable for all temperature treatments suggesting that this phage was remarkably temperature-stable. The same held true for phages M and S for temperatures up to 37˚C, whereas temperatures of 44 and 50˚C resulted in a decrease in infectivity by approx. 1-2 log units.

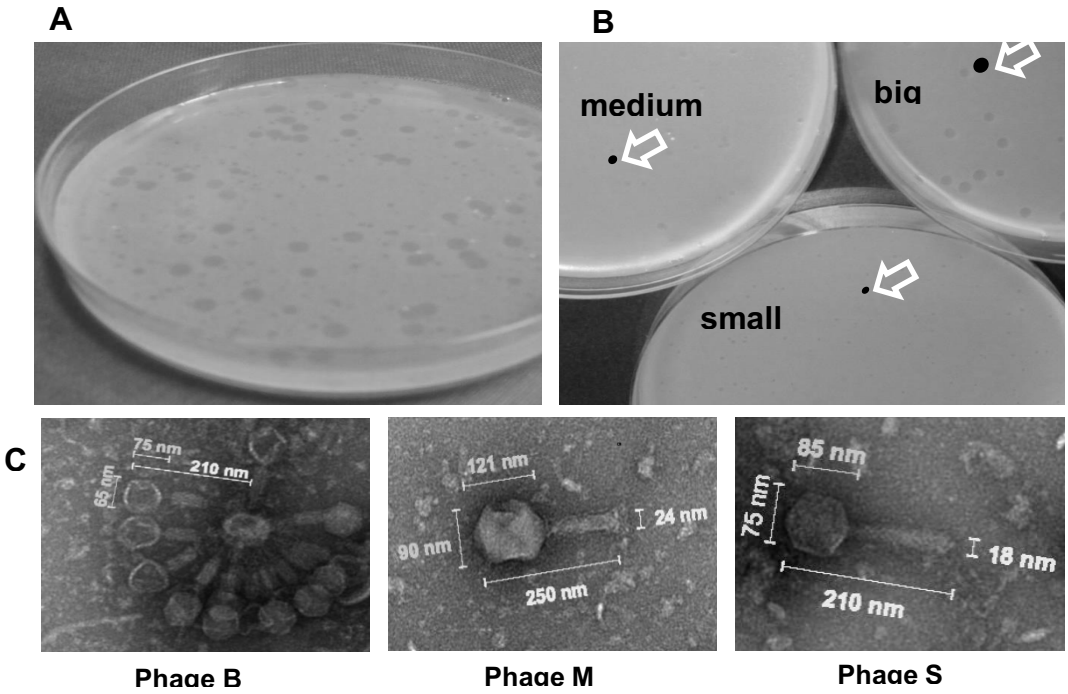

Figure 1. Plaque diversity of *E. coli* specific bacteriophages in brook water receiving treated wastewater effluent. (a) Plaque size diversity from a single isolation with plates being incubated at 20°C. (b) Plaques after multiple isolation rounds. Corresponding phages were designated as: big (B), medium (M), and small (S). (c) Electron micrographic pictures of the bacteriophages. Average dimension of each phage are shown in nm.

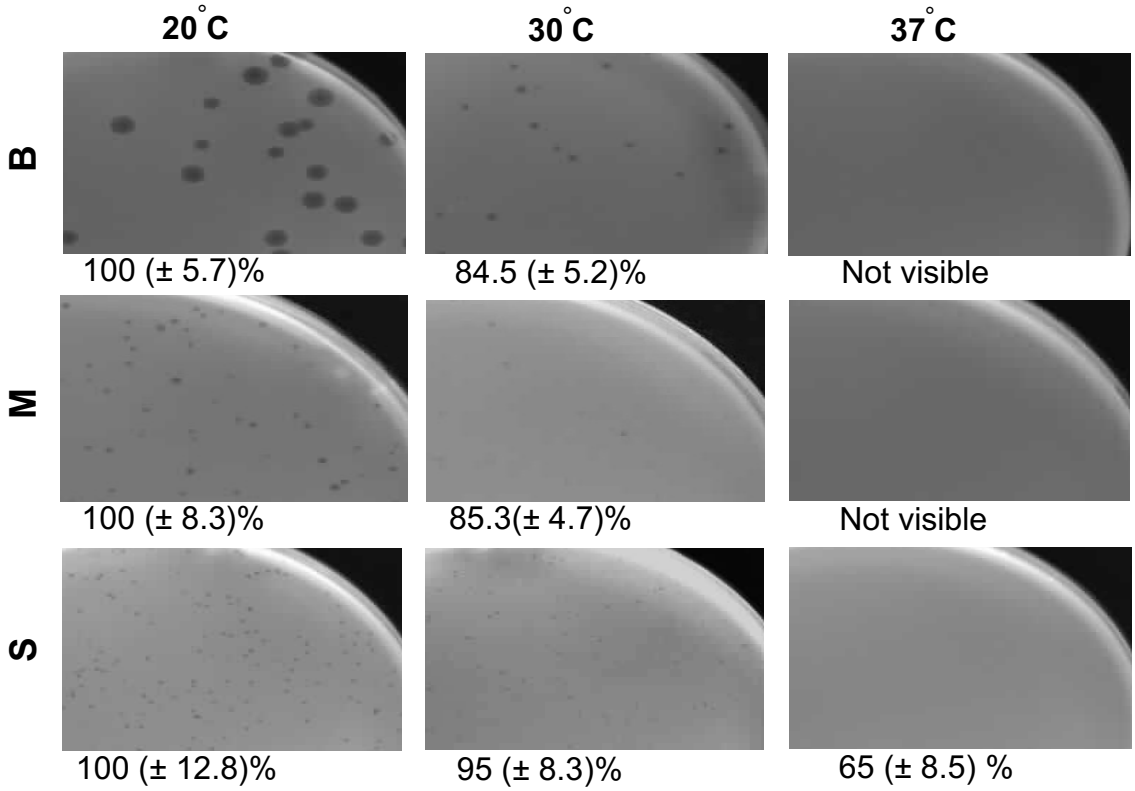

Figure 2. Effect of plate incubation temperature (20°C, 30°C, 37°C) on plaques sizes and numbers for the three *E. coli* phages (B, M and S). *E. coli* was grown at 20°C prior to mixing with phages and overlaying mixtures on TSA. Plates were incubated at indicated temperatures with representative pictures shown. Plaque numbers were normalized and are shown as percentages of the numbers obtained at 20°C. Standard deviations from three independent experiments are shown in brackets.

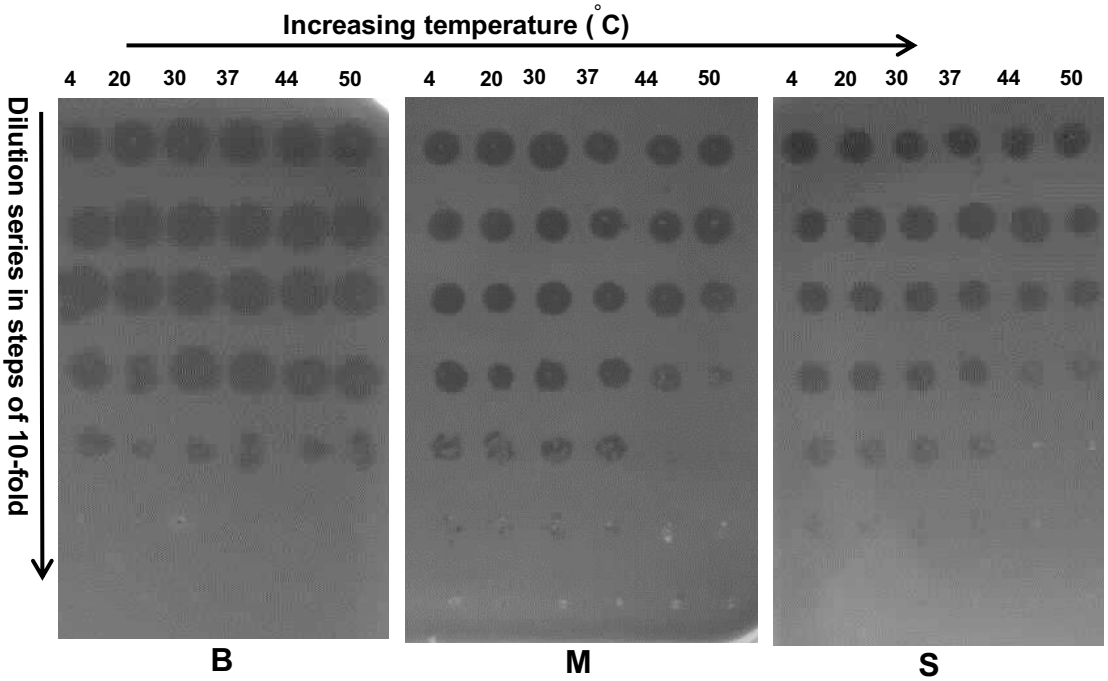

Figure 3. Effect of temperature on infectivity of phages B, M and S after 24 hours of incubation at 4°C, 20°C, 30°C, 37°C, 44°C and 50°C. Temperature-treated phage suspensions were serially 10-fold diluted and aliquots were spotted on tryptic soy agar with pre-plated *E. coli.* Agar plates were incubated at room temperature (20°C) until plaque clearings had reached adequate sizes.

3.3 Susceptibility of E. coli hosts exposed to different temperatures

When exposing pre-grown bacterial host cells (grown at 20°C and suspended in physiological salt to prevent cell replication) to different temperatures (4, 20, 30, 37, 44 or 50°C) prior to phage challenge at 20°C, bacterial susceptibility to lysis greatly depended on their temperature history. Bacteria exposed to temperatures ≤ 37°C were all greatly susceptible to phage lysis with greatest susceptibility (within 12 hours after phage challenge) when exposed to 4 and 20°C (Fig. 4). Bacteria exposed to sublethal heat (41 and 44°C) on the other hand appeared resistant to phage lysis and increased in numbers. No change in optical density was observed for bacteria exposed to 50°C. As no colony counts were obtained, this temperature can be considered lethal to the bacteria with dead cells not allowing phage replication.

3.4 Temperature history of host

Whereas the previous experiment investigated the effect of temperature on pre-grown *E. coli*, we were interested in the susceptibility of bacteria actively grown at different temperatures. For this purpose *E. coli* was grown either at 20 or 37°C prior to mixing with phage B, plating on soft agar and incubation of plates at either 20, 30 or 37°C (experimental design is shown in Fig. 5A). Resulting plaque sizes were greatest when host cells were grown at 20°C and plates were incubated at the same temperature. If bacteria on the other hand were grown at 37°C, equilibrated to room temperature prior to phage addition and the phage-host mix incubated at 20°C, resulting plaques were visibly smaller (Fig. 5B). This result demonstrates that not only does the incubation temperature of the phage-host mix affect plaque size, but also the growth temperature history of the host cells. A similar trend was observed for 30°C, although plaque sizes were generally smaller making the difference less obvious. In agreement with previous data, no plaques were obtained when soft agar plates were incubated at 37°C.

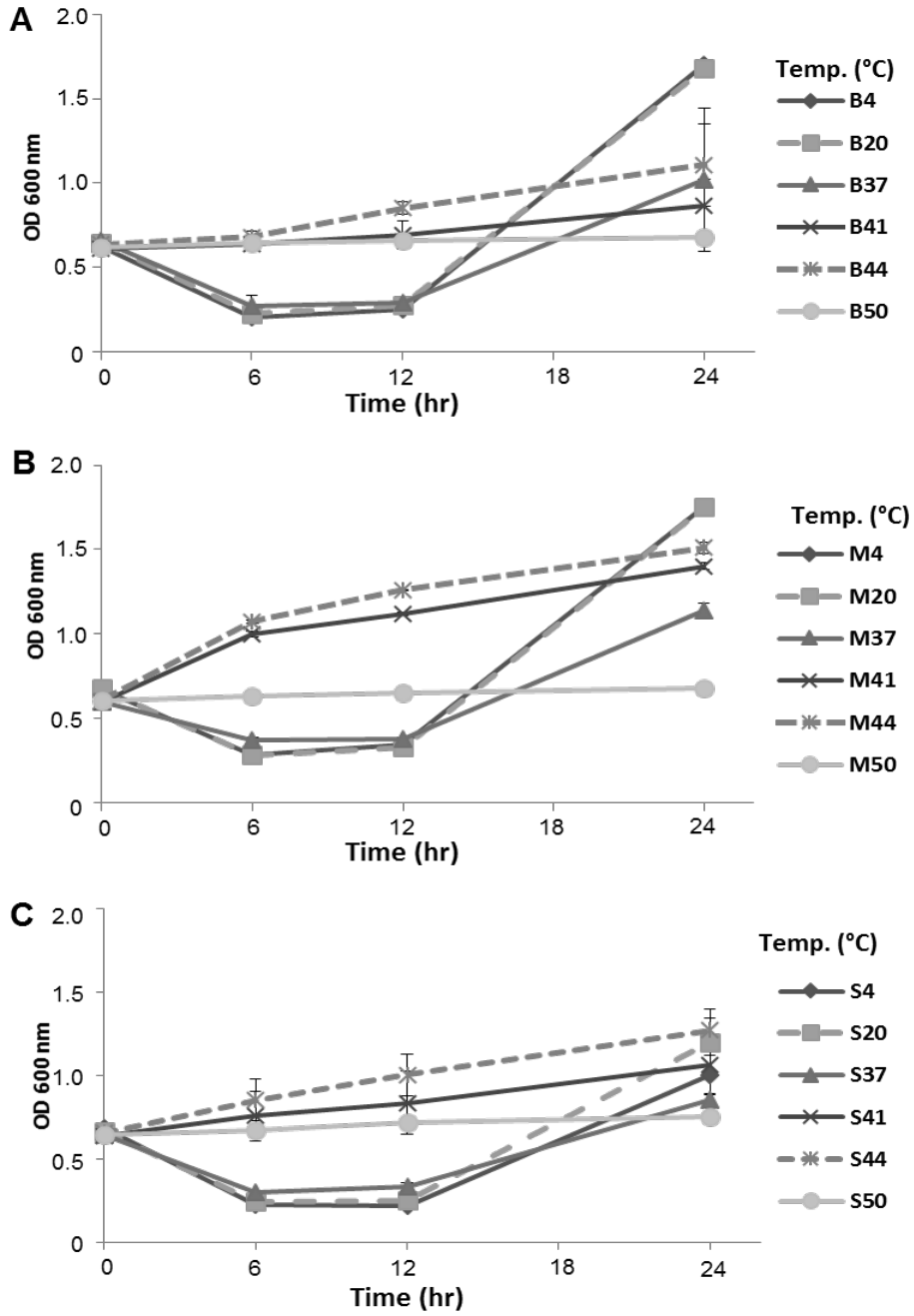

Figure 4. Effect of temperature history of *E. coli* on susceptibility to lysis by phage B (a), M (b), and S (c). Bacteria (OD_{600} = 1) were suspended in PBS and exposed to different temperatures (4, 20, 30, 37, 41, 44 or 50°C) for 24 h prior to resuspension in TSB and challenge with phages B, M or S (MOI = 1) at 20°C. Changes in optical densities were monitored using a microplate reader. Error bars show standard deviations from three independent experiments.

Discussion

The study investigated the effect of temperature on the susceptibility of a clinical *E. coli* isolate to three exemplar environmental phages distinguishable by their plaque size. The fact that temperature-dependence of lysis efficiency was found for three phages from a single sample underlines the importance of this environmental factor. Phage-host mix incubation temperatures that were below the optimal growth temperature of the bacterial host tended to result in bigger plaque sizes and higher plaque numbers. Similar observations have been made previously with dairy starter strains where the optimal lysis temperature was lower than the optimal growth temperature of the bacterial host [4] [5].

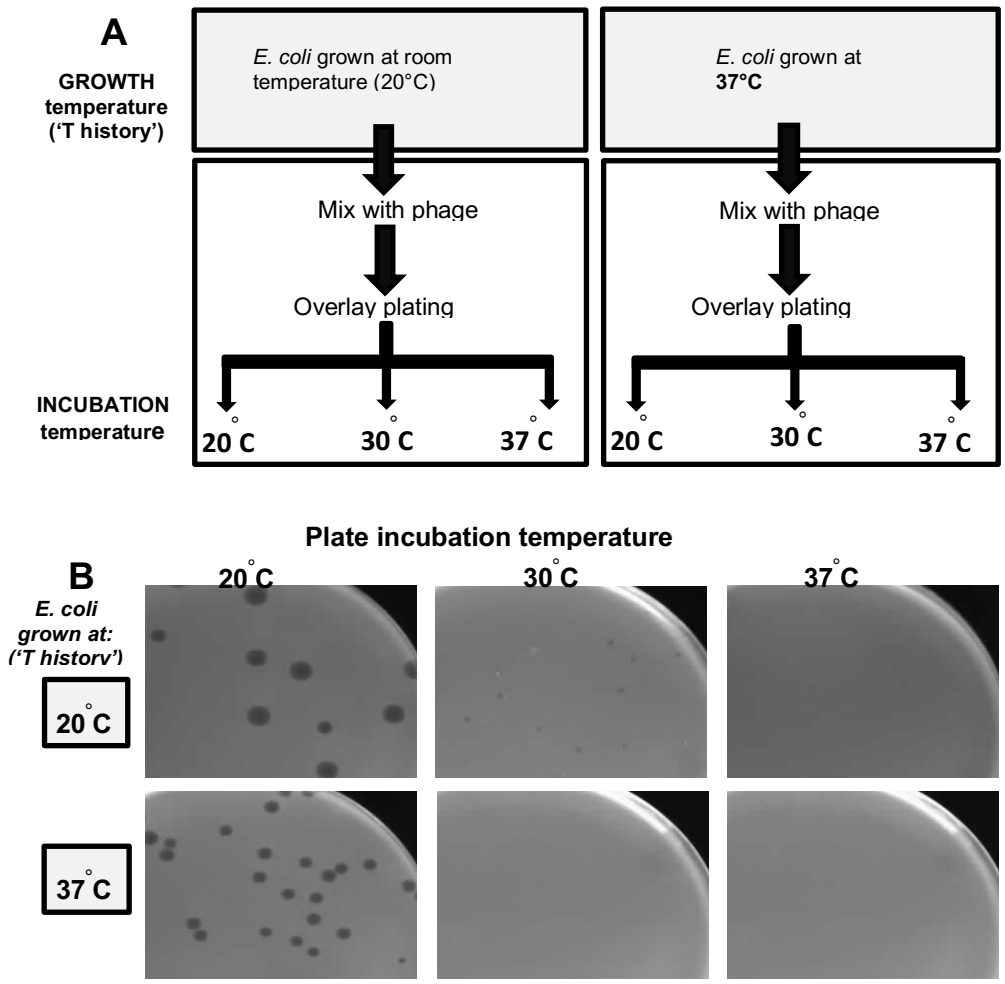

Figure 5. Effect of *E. coli* growth temperature on susceptibility to phage lysis. (a) Schematic presentation of experimental design: *E. coli* cells were grown overnight either at 20°C or 37°C, followed by addition of phage B (MOI=1) and incubation of bacteria-phage mixtures at 20°C, 30°C or 37°C. (b) Plaque sizes obtained after overnight incubation are shown in.

For example, a phage specific for *Lactobacillus casei* did not replicate above 40°C although the bacterial host had a temperature optimum between 37 and 41°C and grew well at 43-44°C [14]. [5] summarized findings that 'certain bacteriophages are unable to multiply at temperatures at which their host bacteria still grow actively' and [4], concluded that 'in many instances the conditions for maximum activity of bacteria and phage are not identical, and the fact that an organism grows most rapidly at a given temperature is not necessarily an indication that a phage race which is able to develop at the expense of that organism will find the conditions most favourable at that particular temperature'. Findings are furthermore in line with the before-mentioned existence of LT, MT and HT coliphages [11]. Based on this classification, the phages isolated in our study would fall into the LT category as they fail to produce plaques when the host-phage mixture is incubated at 37°C (phages B and M) or showed reduced plaque numbers (phage S). The widespread impact of incubation temperature on lysis corroborates the recommendation by other authors [15] that a screening for suitable phages should always be performed under the temperature conditions relevant for the subsequent application to avoid suboptimal results or complete failures.

Our findings suggest, however, that the host-phage interaction is not only dependent on the ambient conditions encountered after mixing bacteria with phages, but additionally by the bacterial temperature history prior to encountering the phages. As for the phages, the exposure to different

temperatures prior to addition to bacteria did not affect lysis efficiency (probably as long as virus stability was maintained). Temperature history was unlikely to be an important factor for the phages, but predominantly for the host cells. The temperature history of fully grown cells produced remarkable differences in lysis susceptibility (Fig. 4). When *E. coli* cultures (grown at ambient temperature, approx. 20°C) were exposed to temperatures ≤37°C prior to being challenged with a phage at 20°C, they underwent a decline in optical density. Exposure to temperature stress in the sublethal range (41 and 44°C) on the other hand resulted in a lysis-resistant phenotype. One reason could lie in the temperature-dependent expression of surface antigens which can serve as phage attachment sites [16]. Also an involvement of the bacterial heat shock regulon could potentially play a role as suggested in a different context by [17]. Authors of this study found that, when *E. coli* were grown at 30˚C for many generations, infected with phage (T2, T4, T6 or T7) and shifted to 42.8-44˚C, 'low to negligible phage numbers were produced' (compared to phage production at 30˚C). If host cells were however adapted to the higher temperature for 8 min before challenge, production of all four phages increased by factors between 3.4 and 267-fold. It needs further analysis to answer whether this observation holds true for more phage-host interactions and potentially to other stresses.

Apart from the temperature that pre-grown host cells were subjected to prior to phage challenge, the growth temperature of host cells was found to modulate lysis efficiency (Fig. 5B). A role of bacterial growth temperature was also reported for *Listeria monocytogenes* [9] although with a reversed temperature effect. Broad host range phages produced plaques only on *Listeria* strain ECII when the bacteria were grown at 37°C, but not when they were grown at temperatures ≤30°C. Bacteria grown at lower temperatures were reported to be resistant regardless of the temperature during phage infection and plate incubation. For the phage-host combinations in our study, on the other hand, phages could still form plaques on *E. coli* grown at 37°C prior to phage challenge (which is a plate incubation temperature that did not permit lysis for the phage used) as long as phage-host mixtures were incubated at lower temperature. The largest plaques however resulted from cultures of *E. coli* grown at 20°C and then incubated at 20°C following phages challenge.

Whilst this study did not aim to elucidate the mechanism responsible for the reported observations, much research will be necessary to understand the underlying reasons. Temperature has been reported to affect phage adsorption, penetration, multiplication [18] and phage performance is strongly dependent on bacterial physiology and metabolism which in turn depends on temperature [19]. As phages do not have a metabolism and therefore no temperature-dependent expression of surface structures, factors determining lysis efficiency might be more limited than for the bacteria although phages seem to be highly adapted to the local conditions in their environment and might have an in-built temperature preference for reversible and irreversible adsorption and replication once mixed with bacteria. As long as phages and their bacterial host co-exist in the same environment for a long time, this specialization appears adequate. The scenario is different however in the case of bacterial pathogens with two distinct life cycles. The temperature in a human or warm-blooded animal host (37˚C) tends to be very different from the temperature encountered, in the environmental life cycle (with water and food being important vehicles of transmission) [20]. It is tempting to speculate that bacteria replicating at 37°C (as prevalent in humans and warm-blooded animals) might, after being released into the environment, be more resistant to phages that are adapted to lower temperatures. The observation that bacteria are not equally susceptible under all conditions would provide them with the possibility to escape predation and add to other defence mechanisms. A change in habitat might sometimes be all that is needed to escape a specific phage although every habitat can be assumed to have its own challenges.

Conclusions

We report here an example of three different coliphages isolated from a single environmental water sample. Temperature of incubation of the phage-host mix greatly influenced both plaque sizes and numbers. The susceptibility of the phage lysis efficiency was very distinct from the temperature stability of the two players suggesting that temperature classification of lysis has to refer to the phage-host mixture as an entity and not to the temperature preferences of phages or bacteria. Apart from the temperature at which the phage-host mixture is incubated and at which lysis occurs, the temperature history of the bacteria was found important. Both exposure of fully grown bacteria to different temperatures or growth at different temperatures prior to phage encounter was found to greatly determine their susceptibility to lysis. The results of these findings have implications for ecological interactions of phages with bacterial pathogens that can spend different parts of their lifecycle at different temperatures. The elucidation of the mechanisms for attachment and phage growth, and bacterial escape warrant further investigation.

Acknowledgements: Ekwu Ameh acknowledged the United Kingdom government through the Commonwealth Scholarship, and the Nigeria government for providing the funding for this project. Also my appreciation to the Department of Biological Sciences and Molecular Biological, Birkbeck University of London were the transmission electron microscopy of the bacteriophages was taken.

Disclosure statement: *Conflict of Interest:* The authors declare that there are no conflicts of interest. *Compliance with Ethical Standards:* This article does not contain any studies involving human or animal subjects.

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