Lysis performance of bacteriophages with different plaque sizes and comparison of lysis kinetics after simultaneous and sequential phage addition

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Abstract

*Background***:** Although bacteriophages see a revival for specifically removing undesired bacteria, there is still much uncertainty about how to achieve most rapid and long lasting clearance.

Materials and Methods: This study investigated the lysis kinetics of three distinct environmental coliphages reproducibly forming different plaque sizes (big, medium and small). Lysis performance by individual phages was compared with the one obtained after simultaneous or sequential addition of all three phages. Kinetics was monitored by density absorbance or by flow cytometry with the latter having the advantage of providing higher sensitivity.

Results: Plaque size happened to correlate with lysis kinetics in liquid suspensions with phages producing big (phage B), medium (phage M) and small (phage S) plaques showing maximal bacterial clearance under the chosen conditions within approx. 6, 12 and 18 hours. Use of a phage cocktail (all three phages added simultaneously) resulted in slower initial lysis compared to the fastest lysing phage with greatest plaque size alone, but showed the longest efficacy in suppression. When adding phages sequentially, overall lysis kinetics could be influenced by administering phages at different time points. The lowest bacterial concentration after 36 h was obtained when administering phages in the sequence S, M and B although this combination initially took longest to achieve bacterial clearance.

Conclusions: Results support that timing and order of phage addition can modulate strength and duration of bacterial suppression and thus influences overall success of phage treatment.

Keywords: phage cocktail, simultaneous phage application, sequential phage application, phage lysis kinetics

Introduction

The increasing resistance of bacterial pathogens to available antibiotics is a major threat to human health. Given the slow pace to develop new drugs there is an urgent need for alternative biocides that are safe for medical applications[1,2]. Alternative biopreservatives are also in demand by the food industry with an emphasis on safety for human consumption[3]. Bacteriophages (phages) are an attractive natural choice as they are highly specific for their bacterial host and do not pose threat to humans. Nature offers a seemingly unlimited diversity of these self-perpetuating entities which naturally evolve with their bacterial targets.

Despite their great promise, science is only beginning to understand how to use phages in the most efficient manner. Overshadowed by the attention that antibiotics received, the efficacy and kinetics of phages as biocidal agents have historically not undergone the required scrutiny[4]. Given the natural diversity, great differences exist in the efficiencies of the different phages to infect their bacterial targets[5]. The strongest focus is on obtaining fast and efficient clearance and on avoidance of the emergence of bacterial resistance. For this reason and to ensure a great host-spectrum, phage cocktails are typically used. Cocktails have been reported to be more effective in reducing bacterial mutation frequency[6].

Alternatively to phage cocktails, individual phages can be administered in a sequential manner. It is currently not clear whether it is best to apply phages simultaneously or sequentially[7]. If bacteria are unlikely to develop multiresistance to different phages, simultaneous application may be considered better[8]following a 'hit them hard' strategy. If multiresistance to individual phages however is likely to evolve relatively fast, it might be beneficial to add individual phages sequentially to control bacterial numbers for longer[7]. In one of the few studies comparing the two different types of phage application, Hall et al. reported that simultaneous phage addition is most effective to clear bacterial infection as

different phages target different bacterial receptors and therefore minimise the probability of cross-resistance[7]. Sequential applications on the other hand are associated with a lower incidence of multiresistance.

In this study, we investigated strategies to achieve highest lysis efficiency based on the use of three phages that reproducibly formed different plaque sizes. Phages giving rise to big, medium and small plaques were referred to as B, M and S with all three belonging to the Myoviridae family. Plaque sizes were correlated with lysis kinetics. Performance of individual phages was compared with the one of a phage cocktail where all the three phages were applied simultaneously. The lysis kinetics of best individual performer and the cocktail were further compared with sequential additions of phages. In the latter case, individual phages with different lysis kinetics were added at different time points to compare lysis speed and sustainability of the overall effect. The overall aim of this in vitro study was to gain more insight into the efficiency of bacterial clearance and the sustainability of the effect when applying distinct phages with different lysis kinetics individually, simultaneously or sequentially.

Materials and Methods

Bacteria and growth conditions

Escherichia coli ATCC 25922, a clinical isolate was used both to isolate phages and as target organism for studying lysis kinetics. Bacteria were grown overnight on tryptic soya agar (TSA; CM0131; Oxoid Ltd Basingstoke, Hampshire, UK) at 30ºC. In experiments running for up to 36 hours, selected samples were plated on selective Membrane Lactose Glucuronide Agar (MLGA; Oxoid, Fischer Scientific, UK) for verification purposes. Green colonies were considered to be *E. coli* colonies. Liquid cultures were obtained by inoculating 10 mL of tryptic soya broth (TSB; CM1016; Oxoid Ltd Basingstoke, Hampshire, UK) contained in 50 mL Falcon tubes followed by overnight incubation for approx. 18 h at 25ºC 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 $CaCl_2*6H_2O$, SIGMA-ALDRICH, Croatia) 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.

Sampling, enrichment and isolation of bacteriophages

Water samples were collected from Chicheley brook (national grid reference: SP94sw; water body ID: GB105033038040) flowing across the Cranfield University campus. Samples were taken at a location where the brook water mixes with treated effluent discharge from the Cranfield University wastewater treatment plant. 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 10 mM $CaCl_2$ to

obtain a final concentration of 5 mM). The mixture was inoculated 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 a 0.22 µm membrane filter (Millex GP, Merck Millipore, Darmstadt, Germany). Appropriate dilutions of enriched sample were plated using the soft agar overlay method (Carey-Smith et al., 2006). Plates were examined for plaques after overnight incubation at room temperature (20ºC ± 2ºC). Plaques of three different sizes were selected and transferred into SM buffer (100 mM NaCl, 8 mM MgS0$_4$*7H$_2$0, 50 mM Tris-Cl, adjusted to pH 7.5), re-suspended and, serially 10-fold diluted for re-isolation. Phages giving rise to big plaques were referred to as big (B) and those giving rise to medium and small plaques as M and S, respectively. Plaque sizes were however dependent on the chosen plate incubation temperature. While differences in plaque sizes were very pronounced at 20°C, s, they got smaller at 30°C. No plaques were obtained at 37°C for the phages B and M.

Purification of bacteriophages

Bacteriophages were isolated three times using a modified soft agar overlay method (Carey-Smith et al., 2006; with some modifications) to ensure purity. A mixture of 100 µL of viral suspension (stock 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 46 - 48ºC in a standard laboratory heat block with wells filled with water for better hear transfer), mixed immediately by gentle vortexing and then distributed evenly over TSA agar plates supplemented with Ca^{2+} or Mg^{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 room temperature (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 20℃ 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 4℃.

Propagation and determination of bacteriophage titres

Phage suspensions were propagated further to obtain higher titre suspensions by infecting 10 mL in 50 mL capacity conical centrifuge tubes (Fisherbrand, Fisher Scientific UK Ltd., Loughborough) *E. coli* culture with phage at a multiplicity of infection (MOI) of 1.0. The mixture was incubated at 20℃ until complete lysis had occurred (typically within 6 – 8h), followed by addition of 8 – 10 drops of chloroform to get rid of remaining bacteria. It was further centrifuged in a 15 mL conical centrifuge tubes (Fisherbrand, Fisher Scientific UK Ltd., Loughborough) at 5,000g for 5 minutes. The resulting phage suspension was filtered through 0.22 µm filter (Millex GP, Merck Millipore, Darmstadt, Germany) and serially diluted in SM buffer. Selected dilutions were subjected to plaque assay using the soft agar overlay method (as described previously) to determine plaque forming units per milliter (PFU ml^{-1}). High titer phage stocks of above 10^9 PFU/mL were stored at 4°C for further experiments.

Transmission electron microscopy

Samples were prepared by pipetting 3 µl of phage suspension (approx. 10^9 PFU ml^{-1}) onto a negatively glow discharged 10 µm thick C-Flat^{TM} carbon grids (400-mesh) followed

by a 1 min incubation. Sample droplets were then partially blotted on Whatman™ quantitative filter paper, Grade 1 followed by application of 3 µl of 2 % uranyl acetate stain solution. After 1 min of staining, excess fluid was removed from the grid surface by positioning the filter in an angle and air drying the sample for approx. 3-5 min. The sample on each grid was then imaged in low dose conditions on a Tecnai 10 transmission electron microscope (FEI company, Oregon, USA) operating at 100 keV. Images were taken at 20 K magnification and captured using a Gatan Ultrascan 4000 4k × 4k CCD camera equipped with an ultra-sensitive phosphor scintillator (Gatan, USA) to produce a final pixel sampling of 11 Angstroms per pixel.

Phage challenge 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 suspension to 10 mL of bacterial suspension (adjusted to an optical density OD_{600} of 1.0) at an MOI of 1 (if not indicated otherwise). Changes in optical densities at 600 nm (OD_{600}) of cell suspensions (due to phage-induced lysis) were measured on a TECAN M200 Pro plate reader. Samples of 1 mL were aseptically 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). Three independent repeats were performed for every phage challenge experiment.

Flow cytometric analysis

For flow cytometry analysis all samples were diluted in 0.1 µm filtered Evian mineral water to cell densities $\leq 10^6$ mL^{-1} to be within the detection range. In order to quantify intact

cell concentrations (ICCs), a 10,000× stock of SYBR Green I (cat. S-7567; Life Technologies Ltd., Paisley, UK) was diluted with dimethyl sulphoxide (Fisher Scientific, Fair Law, NJ) to a 100 × working solution. A dye mixture was made of 100 × SYBR Green I and Propidium iodide (PI) (1 mg mL^{-1}, corresponding to 1.5 mM; cat. P3566; Life Technologies Ltd, Paisley, UK) at a ratio of 5:1, respectively. Volumes of 2.4μL of this dye mixture were prealiquoted into 96 well plates (Corning Costar Flat Bottom Cell Culture Plates cat. nr. 07-200-87; Fisher Scientific, USA) followed by addition of 200 μL of sample (using a multichannel pipette) to give final concentrations of 1× SYBR Green I and 3 μM PI. Once the dyes and sample had been mixed they were incubated for 13 min at 37°C in a shaken plate incubator (Grant instrument Ltd, Cambridge, UK). For the analysis a BD Accuri C6 flow cytometer with a 488 nm solid-state laser (Becton Dickinson UK. Ltd, Oxford, UK) was used. Sample volumes of 25 μL were analysed at a flow rate of 66 μL min-1. All samples were measured in triplicate. The green fluorescence was collected in the FL1 channel at 533nm and red florescence in the FL3 channel at 670nm with the trigger set on the green fluorescence at a value of 1000. For quantification of intact cells the fixed gate described previously by Gatza et al. was used as a template. [10] All data processing was carried out using the accompanying Accuri C6 software and Microsoft Excel.

Statistical analysis

Lytic performances between treatment groups at different time points were analysed using Repeated Measures One-way analysis of variance (RM-ANOVA) using General Linear Models (GLM) followed by post-hoc Tukey HSD post-hoc analysis. Statistics were performed using Statsoft, Inc. (2020) STATISTICA version 13.3 (data analysis software system), with an alpha value of 0.05.

Results

Experiments were performed with three distinct lytic coliphages isolated from a single brook water sample. Phages were differentiated on the basis of the relative plaque size they produce at room temperature (20ºC ± 2ºC). Phages producing big plaques were designated B and those producing medium and small plaques were designated M and S, respectively. Differences between plaque sizes got smaller when incubating plates at 30°C, while 37°C did not lead to formation of visible plaques in case of phage B and M. The three phages were morphologically characterised by transmission electron microscopy and found to belong to the group of Myoviridae, which are double-stranded DNA phages with contractile tails. Phage M has a prolate head, while phages B and S both have icosahedral heads (Fig. 1).

Correlation of plaque sizes with lysis kinetics

Phages B, M and S were mixed with *E. coli* suspensions in a MOI ratio of 1 and lysis kinetics was monitored over a total time of 36 hours using plate reader absorbance measurements. Lysis kinetics were statistically different for all three phages ($p < 0.001$) and correlated with plaque size with the fastest lysis being obtained with phage B, followed by phages M and S (Fig. 2A). The resulting curves were used to define time to maximal clearance (TMC) as a rough indicator of lysis efficiency with TMCs being within approx. 6, 12 and 18 hours for phages B, M and S, respectively. Bacterial recovery (as measured by an increase in optical density reflecting bacterial numbers) was seen fastest in case of treatment with phage B with substantial bacterial regrowth already after 24 hours. Recovered cells were confirmed to be *E. coli* by growth on selective MLGA growth agar. Regrowth of *E.coli* exposed to phages M and S followed with a time delay. Absorbance measurements were in broad consensus with flow cytometric quantification of intact cell numbers (Fig. 2B).

Comparison of phage B with a three phage cocktail

Lysis kinetics of the fastest lysing phage (B, MOI of 1.0) was compared with the performance of a cocktail of all three phages (B, M, S) added simultaneously to an *E. coli* suspension (MOI of 0.33 for each of the three phages with an overall MOI of 1.0). Absorbance measurements suggested a comparable lysis efficiency of the single phage B and the BMS cocktail for the first 12 hours (Fig. 3A). After that time period regrowth was more strongly suppressed in case of the cocktail whereas cell numbers strongly increased when using only phage B. Although lysis kinetics were statistically different ($p < 0.001$) between the single phage and the cocktail, a more detailed picture for the initial lysis efficiency was obtained by flow cytometry. Flow cytometry suggested greater initial lysis after 6 hours in the presence of only one single phage (phage B) compared to the phage cocktail (Fig. 3B and C). Faster regrowth of *E. coli* only exposed to phage B was confirmed also by flow cytometry similar to the results provided by the plate reader. Given the higher sensitivity of flow cytometry for low cell densities, only this analytical method was applied in the following experiment.

Comparison of lysis kinetics of simultaneous addition of multiple phages vs. sequential phage addition

The bacterial lysis of *E. coli* after addition of a single phage (B) and the simultaneous addition of all three phages (BMS) as in Fig. 3 was supplemented with the sequential addition of the individual phages at different time points to bacterial suspensions. The MOI at each single time point of addition was 1 relative to the initial concentration of bacteria prior to phage addition. In the case of sequential additions, an additional phage was always added at the time that represented the end of maximal clearance time of the previous phage (according to TMC values indicated in Fig. 2). The time scale of the phage addition is shown in Fig. 4A.

Fastest initial lysis was obtained when only one phage (B) was mixed with the bacteria (Fig. 4B). Consistent with previous data, lysis kinetics was in the order of phage B > M > S for the mixtures receiving sequential phage addition. The *E. coli* suspensions challenged with the cocktail (with each phage at an MOI of 0.33) showed intermediate lysis compared to the samples initially challenged with a single phage (at an MOI of 1.0).

Whereas phage B alone led to the fastest lysis within the first 6 hours, all combinations with three phages (whether applied simultaneously or sequentially) performed better at later stage ($\geq$ 24 hours) as bacterial regrowth was more efficiently suppressed. Comparing simultaneous and sequential phage addition, no generalizations were possible. Despite differences in initial lysis kinetics (< 24 h), combinations $B_0M_0S_0$, $B_0M_6S_{18}$, and $M_0B_{12}S_{18}$ resulted in comparable bacterial titers on the log scale after 36 hours. All differences in lytic performances were statistically significant (p < 0.001).

Corresponding flow cytometric density plots of end point bacterial populations obtained after 36 h are shown in Fig. 4B. They show that among these three combinations the least intact bacteria were obtained after 36 hours for those combinations, showing slower initial lysis ($B_0M_0S_0$ and $M_0B_{12}S_{18}$). The phage combination resulting in most sustainable suppression of *E. coli* after 36 h was the sequential cocktail $S_0M_{18}B_{30}$, however this combination also led to slowest lysis in the first 12 hours.

Data from multiphage experiments suggest overall that the timing of phage addition (using the phages with different lysis kinetics) allows for a choice between fast initial and a longer lasting effect under the laboratory conditions chosen. For a fast effect the phage with the greatest plaque size proved best in this example, whereas sequential additions with slower lysing phages proved beneficial to obtain a more lasting effect. However no combination could prevent regrowth in the long term under experimental conditions.

Discussion

In this study we investigated the lytic performance of three distinct lytic phages that gave rise to different plaque sizes. Phages were applied individually or in different combinations in terms of order and timing. For the isolated phages, plaque sizes on plate (representing a semi-solid state) happened to correlate with lysis rates in bacterial liquid suspensions. Phage B producing the largest plaques was able to destroy the bacteria at the fastest rate, followed by the phages producing medium and small plaques. This relationship is not surprising as among the factors determining plaque size (for an overview see Gallet et al.[11]), some are shared in common between semi-solid and liquid phage propagation including adsorption rate and burst size (given the identical bacterial host, temperature, and osmolarity). Diffusion rates, bacterial densities and bacterial metabolic rates (influenced by nutrient availability, cell density and oxygen availability) on the other hand can be very different between agar and well-mixed broth[12], which might explain why this correlation is not always observed. When studying the effect of three different phages on *E. coli* 0157:H7 with two phages producing pinpoint plaques (0.5 mm) and one producing medium-sized plaques (3 mm), O'Flynne for example reported faster and more efficient lysis for the two phages producing smaller plaques.[13] Despite plaque size being one of the most important phage traits, questions remain how this characteristic can be used for optimal choice of phages for biocontrol applications. For liquid phage applications,phage kinetics might be ideally determined directly in liquid suspensions. If applied in a biofilm context or for phage therapy in vivo, the corresponding relevant conditions would be more relevant to assess lytic phage performance.

      The central research question of this study however was to compare the lytic performances of three individual phages with the one of multiple phages that were applied either simultaneously in form of a cocktail or sequentially at different time points in regard to lysis efficiency and the sustainability of this effect. One of the first questions was which

diagnostic assay to apply to measure lytic activity. As the plaque assay is not compatible with high sample numbers and subject to variation (depending on the experimenter, timing and experimental conditions)[7], both optical density measurements and flow cytometry (in combination with live-dead staining) were applied. Both methods are substantially faster for laboratory experiments with a given bacterial target strain than the tedious plaque assay. Although grossly in agreement, a difference in sensitivity between the two methods became visible when comparing the lytic performance of phage B with the one of the cocktail containing all three phages (Fig. 3). Whereas density measurements suggested a highly comparable performance up to 12 hours, flow cytometric measurements showed a greater decrease in the concentration of intact bacteria when exposed to phage B alone. The reason lies the greater sensitivity of flow cytometry. Whereas the limit of detection of a plate reader in combination with the comparable LIVE/DEAD BacLight™ (comprising staining with SYTO9 and propidium iodide) was reported to have a limit of detection in the range between 10^6 and 10^7 cells mL^{-1} [14]. The detection limit of flow cytometry can be as low as 100 cells mL^{-1} (depending on the cleanliness of the machine prior to analysis as background signals strongly interferes with the detection limit). The better limit of detection of flow cytometry allowed the observation that the application of the best performing phage alone resulted in a greater decrease in intact cell concentrations than the cocktail with the same overall MOI within the first 6 hours after phage application (Fig. 3). Both methods suggested on the other hand a shorter duration of lysis efficacy in the monovalent case reflected in a faster recovery of bacterial numbers. Due to this superior resolution in the low cell density range, flow cytometry was used for subsequent experiments.

The results show that application of high numbers of a fast-lyzing phage (MOI=1) results in fastest bacterial clearance compared to a phage cocktail with a comparable overall MOI split among the distinct phages (MOI of each phage of 0.33). In other words, a phage

cocktail is not necessarily the best choice, if the emphasis is on fast bacterial clearance. On the other hand, the application of multiple phages show greater suppression at 24 or 36 hours after phage addition. The synergistic benefit of multivalent cocktail in regard to delaying resistance is generally accepted especially if phages target different bacterial receptors[15-17]. When for example comparing the impact of a single phage and a cocktail of four phages in the biocontrol of *Campylobacter jejuni* in broilers, the application of the cocktail was reported to have only a small advantage over the single phage treatment in regard to bacterial lysis potential, however the cocktail delayed the emergence of phage resistance[18]. The benefit of cocktails in regard to suppression of resistance was also reported for *Escherichia coli* O157:H7[19]. On the other hand the application of a phage cocktail has also the potential to lead to a greater general resistance as shown in the case of *P. aeruginosa* challenged with up to five different phage types. When testing the resistance of bacterial clones sampled at the end of the challenge experiments and newly challenged with ancestral phages, results suggested that overall resistance was greater when bacteria had been challenged in the first round with a greater number of phage types.

Despite questions over of the extent and kinetics of bacterial resistance, multiphage treatment is typically seen advantageous over monovalent applications due to a broader host range[20] and potentially faster killing of bacterial targets[21]. As it is however currently unclear how multiple phages should be best applied and how the individual cocktail members should be selected, the outcome of phage applications have a poor predictability[22]. One of the few studies on this aspect was the comparison of populations of *P. aeruginosa* after addition of single phages and multiple phages applied either in a simultaneous and sequential fashion[7]. The efficiency of application was assessed *in vitro* (over 12 transfer cycles with aliquots being transferred every 24 h into fresh medium) and *in vivo* using wax moth larvae infected with *P. aeruginosa*. The outcome of the study was that the effect of multiple phages was

more sustainable than individual phages. Comparing the different strategies for multiple phages, simultaneous addition was reported to be consistently equal or superior to sequential addition for clearing bacteria. Authors however pointed out that sequential strategies can be devised that are just as effective as a cocktail and that sequential applications might be associated with a lower incidence of multiresistance. In our experiments, although not employing transfers over multiple replication cycles, a bespoke strategy was chosen for sequential applications by adding the second and third phage at time points that defined the end of maximal lysis of the previous phage. Looking at the performance after 36 hours, the best overall suppression in bacterial numbers was obtained by sequential addition of S, M and B phage ($S_0M_{18}B_{30}$; Fig. 4). Other sequential combinations showed a comparable performance as the phage cocktail after 36 hours in our experiments. Our findings are overall in good agreement with the statement by Hall et al.[7] that sequential strategies can be equally effective or potentially better over longer time scales.

Comparing the lytic performance of the phage combinations in our study, one of the eminent conclusions is that fast initial lysis comes at a cost of relatively poor sustainability. If bacteria got lysed fast, the duration of the effect was shortest due to bacterial recovery. On the other hand, a delay in lysis translated into a longer lasting suppression of bacteria. We did not succeed in our experimental setup to achieve fast initial lysis and at the same time a long-lasting suppression of bacterial growth. It has to be acknowledged that the experiments as performed by us and other researchers conducting this type of laboratory experiments are bound to be relatively artificial as they involve working with a single bacterial species at high cell densities in a closed system batch experiment. Typically bacterial recovery occurs fast under these laboratory conditions within 24 hours or less[7,13,23]. In sharp contrast to such laboratory experiments, real world applications are characterized by a large abundance of different bacterial species in low concentrations translating in high MOIs for the specific

bacterial target. Also nutrient availability does not undergo such a dramatic change in composition and concentration in real world applications as in a closed vial. It therefore remains to be seen whether our observations also hold true at relatively higher MOIs for specific bacterial targets in complex microbial populations in real-world applications where lysis performance and sustainability of the effect can be very different from a laboratory batch setting.

The knowledge of phage kinetics and growth rate is probably one important factor in the selection approach with other criteria to be added. This is in agreement with a study comparing six highly distinct *Pseudomonas aeruginosa*-specific phages from two different environmental water sources on the mean survival time (MST) of infected *Drosophila melanogaster*[24]. Neither adsorption rate, burst size nor lysis time significantly correlated with MST. The only phage trait with significant correlation and best predicting value for therapeutic success was the phage growth rate (as an indicator of phage fitness) as determined in vitro. The growth rate can be seen as a proxy for the bacterial clearance rate used in this study. The authors point out that the in vitro growth rate is a function of individual phage traits. A phage with a high adsorption rate for example may not grow as fast as a phage with a slightly lower adsorption rate, but a much shorter lysis time. Only the sum of all traits eventually determines the overall in vitro growth rate and eventually the fitness.

Interestingly the timing and order of addition of multiple phages in a sequential order have been reported to modulate the upcoming of bacterial resistance and thus the long-term efficacy of phage therapy[25]. A phage pair administered sequentially in the reverse order had different impacts on evolving resistances. The authors stated that the 'timing and the order of phage exposure determined the strength, cost, and mutational basis of resistance evolution'[25]. Thus choosing the right strategy when adding multiple phages can be seen as a promising opportunity for optimizing overall success of phage treatment.

Conclusions

The study performed with phages with different lysis kinetics led to the following observations: (A) a single phage with the fastest lysis kinetics can outperform a phage cocktail in regard to velocity although the cocktail is likely to achieve a more sustainable bacterial clearance, (B) all multivalent applications had a more long-lasting effect than the fastest single phage and (C) certain sequential phage applications can in some cases produce a more sustainable result than the cocktail. The choice to be made in the current example was between fast clearance and sustainability of the effect, no phage application fulfilled both parameters at the same time. It remains to be seen whether a smart, application-tailored choice of sequentially added phages in a real-world scenario could meet both requirements of rapid clearance of the bacterial target and a sustained regrowth suppression of surviving cells. Phage growth rate or time to clearance under conditions that are relevant for the intended application might however be a valuable criterion for the phage treatment strategy in regard to the order and timing of the addition of phages.

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Figure Legends

FIG 1. Electron micrographic pictures of *E.coli* specific bacteriophages (a) phage B, (b) phage M, and (c) phage S. Dimensions of each phage are shown in nm

FIG 2. Comparison of bacterial lysis behaviour after exposing *E. coli* to three different phages producing distinct plaques sizes at room temperature: Big (B), Medium (M), and Small (S). **(A)** Lysis was determined by measuring bacterial optical densities at 600 nm (OD_{600}) at indicated time points up to 36 h. Times to maximal clearance (TMC) indicate time intervals in which optical densities reached minima. Error bars indicate standard deviations from three independent repeats. **(B)** Changes in relative concentrations of intact bacteria over indicated times relative to the intact cell concentration at time point zero together with corresponding standard deviations from three independent repeats.

FIG 3. *E. coli* lysis kinetics obtained after infection with a single phage (phage B) and after simultaneous addition of three different phages (B, M and S) in the form of a cocktail. **(A and B)** Comparison of two cultivation-independent methods based on (A) the measurement of the decline in bacterial optical density at 600 nm (using a microplate reader) and cell concentrations of fluorescently labelled intact bacteria (using flow cytometry) at different time points. **(C)** Flow cytometric dot plots of bacteria-phage mixtures and a bacterial control suspension without phage after staining with SYBR Green I and PI. Intact cells appear within the gated area (dotted lines). Error bars indicate standard deviations from three independent repeats.

FIG 4. Effect of the time of phage addition on lysis kinetics and the duration of lysis efficacy. **(A)** Experimental strategy to add phages to an *E. coli* suspension. Subscript numbers indicate the time point in hours at which a particular phage was added. Time zero indicates the beginning of the experiment. In case of sequential additions, phage 2 and 3 were always added at the time point of maximal lysis of the previous phage. **(B)** Concentrations of intact *E. coli* cells after addition of different phages at different time points as measured by flow cytometry. A sample without phage addition served as a control. Error bars indicate standard deviations from three independent repeats. Flow cytometric (FCM) plots obtained after 36 h are shown with intact bacteria being located in the gated areas.
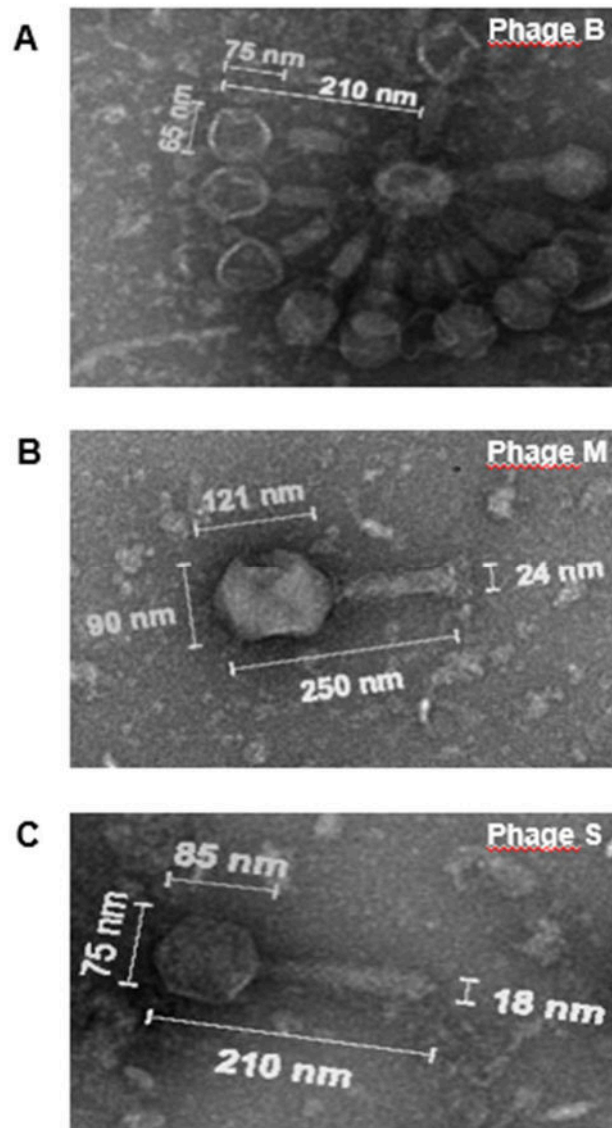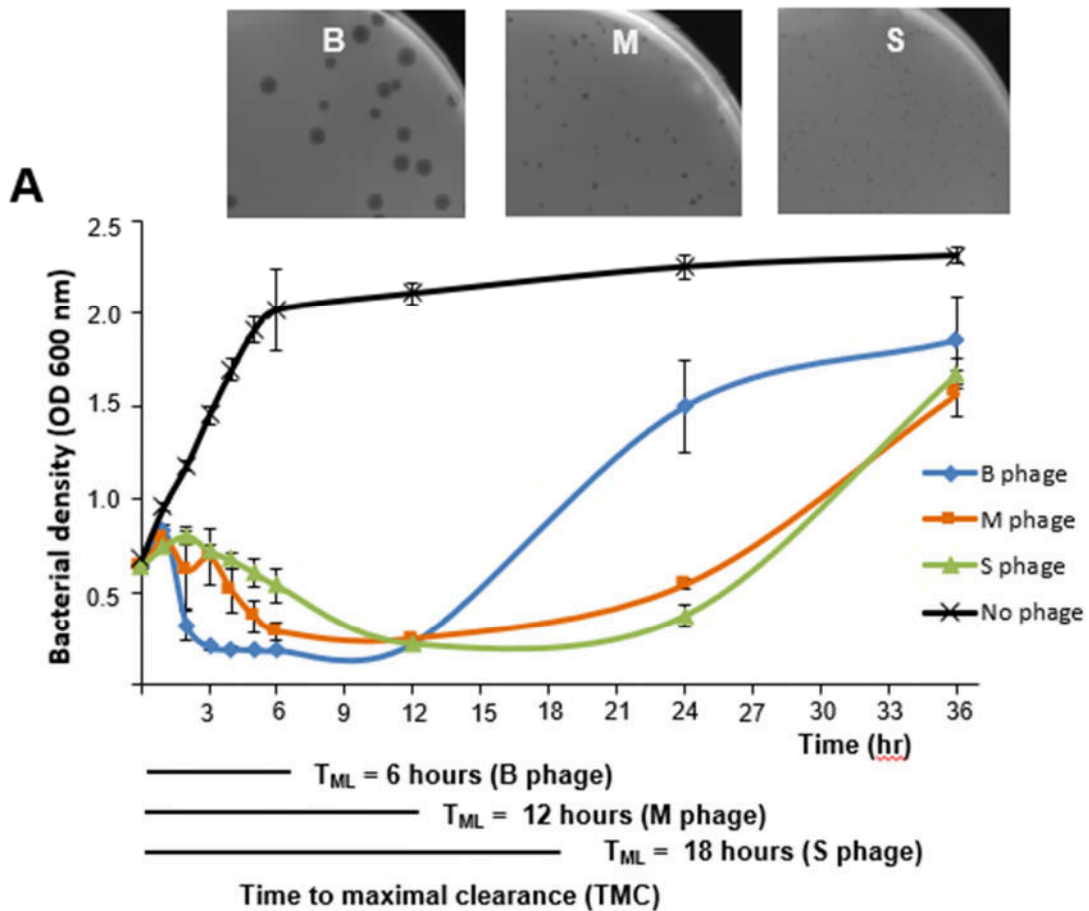
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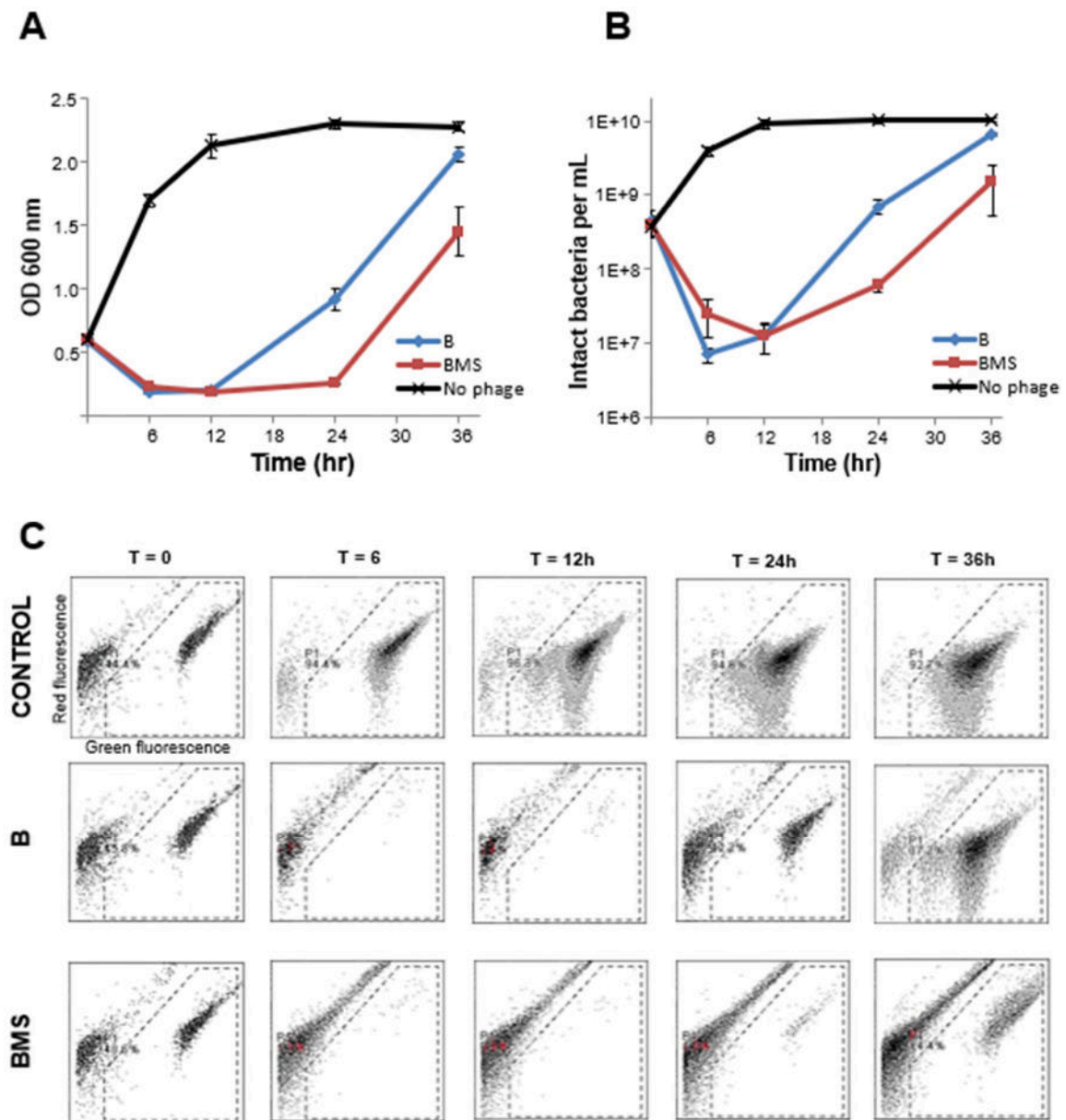
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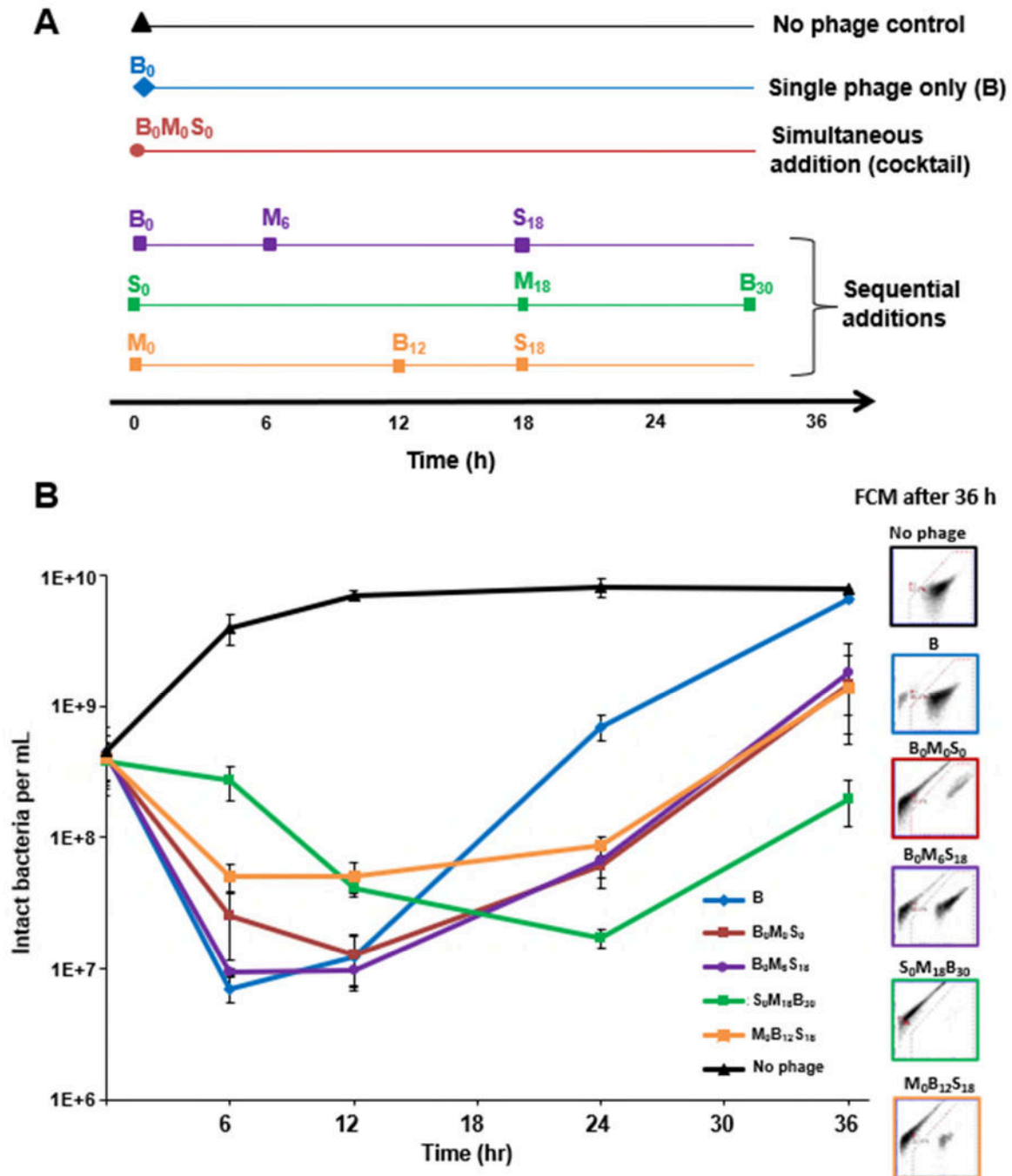
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