Real-time quantitative PCR to discriminate and quantify lambdoid bacteriophages of Escherichia coli K-12

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Quantification of bacteriophages by real-time quantitative PCR (qPCR) is an interesting alternative to the traditional plaque assay. Importantly, the method should in principle be able to discriminate between closely related phages that are indistinguishable by most other means. Here, a method is presented that employs qPCR to discriminate and quantify ten closely related lambdoid phages of Escherichia coli str. K-12. It is shown that (1) treatment of samples with DNase efficiently removes non-encapsidated DNA, while the titer of plaque forming units is not affected, (2) individual phage types can be accurately quantified in mixed lysates, and (3) the detection limit corresponds to that of a plaque assay. The method is used to quantify individual phage types that are released from lysogens that carry up to three different prophages.

Introduction

The infection of a bacterial cell by multiple, different bacteriophages (phages) constitutes an important part of phage ecology that influences phage evolution in many ways.1-3 To study interactions among phages, methods are required that can discriminate and quantify different phage types in the same sample. The double agar overlay assay,6 although considered to be the gold standard for phage quantification, falls short of what is required here. Its capability to differentiate phages is limited as it depends on differences in phenotypic markers such as plaque morphology or host range of the phages to be distinguished.6 Real-time quantitative PCR (qPCR) is an interesting alternative and has been successfully used to enumerate phages as well as other microparasites.7-10 Concerning phages, current applications of qPCR fall broadly in two groups, neither of which requires a highly specific assay. Either the specificity of the method is not an issue as samples are expected to contain only one type of phage, or the method has been deliberately adapted to be unspecific such that a broader group of phages is detected.11-13 Here, a method is presented where qPCR is used to achieve the opposite, namely the discrimination of closely related phages in mixed lysates.

To develop and validate this method, a set of ten lambdoid phages of Escherichia coli str. K-12 is used. These phages have been used previously to study the competitive interactions that occur when two prophages are induced in the same lysogenic cell.3 Pairs of these phages can be distinguished by differential plating on bacteria that exclude one phage by its receptor specificity or immunity group. However, this approach fails to discriminate most combinations of three or more phages. This can be overcome by a qPCR approach where individual phages types are specifically targeted.

The assay is based on an earlier protocol,9 a modified version of which has been used previously to quantify variants of phage λ that did not produce plaques or whose plaques were difficult to count.14 It consists of two steps, an initial treatment with DNase to remove phage DNA that is not enclosed in capsids (which would cause an overestimation of the phage titer), and the qPCR itself. While the method performs very robustly, its extension to achieve the discriminatory power to accurately quantify individual phages in mixed samples has never been explored and required further experiments for validation.

Together, these experiments show that the assay detects all viable phages in a sample and clearly discriminates individual phages in mixed lysates. The good correspondence to the plaque assay suggests that the method can be used by digital PCR15 for absolute quantification without an external standard and for the quantification of phages which are difficult or impossible to quantify otherwise (e.g., because they form no or barely visible plaques, or no cells suitable for plating are available).14,16

The method is used to quantify individual phages in lysates that were obtained from the induction of lysogens containing either one, two, or three different lambdoid prophages. Specific pairwise combinations of these phages have previously been shown to
str. K-12 substr. MG1655. *E. coli* was grown in lysogeny broth (LB; 10 g l\(^{-1}\) tryptone, 5 g l\(^{-1}\) yeast extract, 1 g l\(^{-1}\) NaCl). For plates, 1.5% agar was added to LB and autoclaved before use. For liquid cultures, LB was titrated to 0.5 McFarland standards and 100 μl of culture was added to 10 ml of LB. Lysates were stock frozen at -80°C. The E. coli strain M4287) at a concentration of 5 × 10\(^{10}\) CFU/ml in LB was used in this study. Prophages were induced with mitomycin C (Sigma, St. Louis, MO, USA) at a concentration of 5 μg/ml for 30 min at 4°C. Prophages were induced by adding 5 μg ml\(^{-1}\) mitomycin C and continuing incubation for approximately 4 h at 37°C. Dilute cultures 1/500 into fresh LB and bring back to exponential growth by incubation for 2 h under continued shaking at 37°C. Induce lysogens by adding 5 μg ml\(^{-1}\) mitomycin C and continue incubation for approximately 4 h at 37°C. Dilute lysates 1/10 in SMG, add a drop of chloroform, and centrifuge at 3700 g for 10 min. Store lysates at 4°C.

DNase treatment was performed with RQ1 RNase-free DNase I (Promega, M6101), using 10X reaction buffer (Promega) containing 400 mM TRIS-HCl pH 8.0, 100 mM MgSO\(_4\), and 10 mM CaCl\(_2\), and stop solution (Promega), containing 20 mM EGTA pH 8.0.

**Materials and Methods**

Materials. *Bacteria, phages, and reagents.*

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**Materials.** *Bacteria, phages, and reagents.* The phages used were all lambdoid phages of *Escherichia coli* str. K-12 and were described elsewhere (Table 1). They all belong to separate immunity groups, except for phages mEp234 and mEp332, which share the immunity group of phage λ. All phages were kept as prophages in *E. coli* str. K-12 subsp. MG1655.

Bacteria were grown in lysogeny broth (LB; 10 g l\(^{-1}\) tryptone, 5 g l\(^{-1}\) yeast extract, 10 g l\(^{-1}\) NaCl). For plates, 1.5% agar was added, for top agar, 0.7%. Lysates were stored in SMG (0.1 M NaCl, 8 mM MgSO\(_4\), 50 mM TRIS-HCl pH 7.5, 0.01% gelatin) at 4°C. Prophages were induced with mitomycin C (Sigma, M4287) at a concentration of 5 μg/ml.

**METHODS AND PROTOCOLS**

**Preparation of phage lysates.** Inoculate single lysogenic colonies in LB and grow under continued shaking overnight at 37°C. Dilute cultures 1:500 into fresh LB and bring back to exponential growth by incubation for 2 h under continued shaking at 37°C. Induce lysogens by adding 5 μg ml\(^{-1}\) mitomycin C and continue incubation for approximately 4 h at 37°C. Dilute lysates 1/10 in SMG, add a drop of chloroform, and centrifuge at 2700 g for 10 min. Store lysates at 4°C.

**Removal of non-encapsidated DNA.** Add 2 μl DNase I (2 units) and 1.3 μl 10X reaction buffer to 10 μl lysate and incubate for 60 min at 37°C. Inactivate DNase by adding 1.5 μl stop solution.

**Primer design.** Primer3 software as included in Geneious Pro v5.5.1 was used together with sequence data of all phages (Refardt D., unpublished data) to select primer pairs that specifically target selected phages (Table 1). Annealing temperature was set to 60°C and product size to ~100 bp. Primers were ordered from Microsynth (Balgach, Switzerland).

**Instrument settings and data analysis.** Amplification reactions were performed with a 7500 Fast Real-Time PCR System (Applied Biosystems) using default settings including a melt curve. Cycle threshold (ct) values, the number of PCR cycles required to amplify a target to a detectable level, were calculated at a normalized fluorescence emission intensity threshold of ΔRn = 0.1, which corresponded to approximately 5% of the maximum intensity. Statistical analyses were performed using R statistical software version 2.11.0.

**Data on viral diversity in various environments accumulate at a staggering pace,** and it is important to test whether concepts from ecological and evolutionary theory can be applied to the processes that give rise to this diversity. Another field where the application of qPCR is promising is phage therapy, the treatment of bacterial infections with phages. The ability to discriminate between individual phages will allow better understanding of phage population kinetics when phage cocktails are used instead of single phages are used.25-27

Table 1. Primers that were validated in this study and the phages against which they are targeted.

| Name          | Sequence                      | Target phage | Target sequence | Product size |
|---------------|-------------------------------|--------------|-----------------|--------------|
| qPCR Mδ22     | 5’-TCGCGCAGGCTGCACTAAACATGG-3’ | mEp222       | d               | 96 bp        |
| qPCR MδEp043  | 5’-ACCACGGGAGACATGTCAGG-3’    | mEp043       | d               | 86 bp        |
| qPCR MδEp234  | 5’-GGATAGGCGGCTTAGGCG-3’      | mEp234       | n               | 96 bp        |
| qPCR MδEp235  | 5’-GCCATTCTGACTGTCGAGAT-3’    | mEp235       | n               | 126 bp       |
| qPCR MδEp25b  | 5’-CAGCGCCGCTTCCGAGATC-3’     | mEp25b       | n               | 120 bp       |
| qPCR MδEp25   | 5’-CCGACGGCTGACTGTCGAGAT-3’   | mEp25        | o               | 120 bp       |
| qPCR MδEp31  | 5’-AACAGCCGCGCTGACTGTCGAGAT-3’ | mEp31       | d               | 105 bp       |
| qPCR MδEp26  | 5’-TGCTACGCGAGACATGTCAGG-3’   | mEp26        | d               | 96 bp        |
| qPCR Lambda   | 5’-CTGCGCAGCGGCTGACTGTCGAGAT-3’ | mEp332     | n               | 120 bp       |
| qPCR Phi80    | 5’-GACACCGCAGGCTGACTGTCGAGAT-3’ | 080        | o               | 120 bp       |
and incubating for 30 min at 95°C. Heating also opens phage capsids and makes DNA accessible for amplification. Dilute treated lysates 10-fold in H2O and store at -20°C.

Quantification by qPCR. Amplify samples in 10-μl reaction volumes including 300 nM of each primer, 1× SYBR Green PCR master mix (Applied Biosystems, 4309155), and 1 μl template.

After overnight incubation of cultures, the complete quantification process can be done within one day. Multiple samples can be efficiently processed in parallel by growing cultures in 96-deep-well plates (add a glass bead to each well to ensure proper mixing of cultures and seal plates with breathable film).

Validation of the protocol. Capsid stability. An experiment was conducted to test whether treatment of lysates with DNase causes a change in the titer of plaque forming units. From each phage, 18–24 separate lysates were obtained and quantified using a plaque assay. Lysates where then treated with DNase and quantified again with a plaque assay before the final heat-inactivation step.

Significant (p < 0.05, Wilcoxon signed-rank test) changes in phage titer were observed in three of ten phages (Fig. 1). Of these, the largest change (+25%) occurred in phage mEp332. This change is moderate when compared with the inter-assay variance, which is typically larger. It is concluded that the observed effect is not relevant for quantification.

Efficiency of the DNase treatment. The amount of phage DNA in lysates that is not enclosed in capsids was measured by amplifying 3–6 lysates per phage with qPCR both before and after DNase treatment. Ct values were consistently higher in treated than in untreated samples (Fig. 2).

Samples of phage mEp332 showed the largest difference (Act = 7.1), which corresponds to a 100-fold excess of non-encapsidated phage DNA relative to DNA in virions (assuming an amplification efficiency of 95%).

To estimate DNase efficiency, lysates were kept at 95°C for 30 min prior to DNase treatment. When these samples were quantified with qPCR, their ct values were found to be at least 8.4 cycles above those of untreated samples (Fig. 2). Thus, DNase removes at least 99.6% of all accessible DNA (assuming an amplification efficiency of 95%). Together, the results confirm that treatment with DNase efficiently removes non-encapsidated DNA from lysates. Using the numbers obtained, the worst possible case is an overestimation of the titer of phage mEp332 by 3% due to residual non-encapsidated DNA.

Primer specificity. All primers were tested in replicate amplification reactions against lysates of all phages and water controls. Generally, primers were found to only amplify the respective target sequence. After controlling for differences in melting temperature (using a difference of ≥ 1°C from that of the target sequence as criterion for exclusion), 18 of 196 negative controls yielded detectable amplifications that would have been accepted as positive results had they occurred in samples with
unknown content. Typically, these arose very late (ct > 35, which corresponds to less than two copies of the target sequence) and inconsistently among replicates. The exception were primers qPCR_mEp235 and qPCR_mEp235b, which very weakly cross-amplified samples of phages λ, mEp332, and mEpX1, and λ, mEp213, and mEp332, respectively. Ct values of these cross-amplifications were all above 30, which corresponds to less than 10 phages. Therefore, these cross-amplifications cause a negligible bias in the quantification of phages. However, detections of very low numbers of phages (in particular phage mEp235) in samples of unknown content must be interpreted with the appropriate caution.

Amplification efficiency. Lysates from all phages were quantified with plaque assays, treated with DNase and then serially diluted down to an expected number of 0.01 phages in the amplification reaction. Standard curves were constructed by amplifying at least four replicates per dilution step (Fig. 3). Amplification efficiencies, estimated by linear regression, were found to be between 87 and 102% and were estimated using the data that are connected by a solid line in Figure 3. This was done to avoid overestimating amplification efficiencies, which occurs when dilution steps are included that contain replicates where no phages were detected.

Detection limit. If qPCR is congruent with a plaque assay, a single phage genome per reaction defines the largest obtainable ct value (horizontal dashed lines in Figure 3). When this limit is approached, variation among replicates increases as the actual number of phage genomes follows a Poisson-distribution. Five percent of all samples with an expected number of three phage genomes will be empty. Ten percent of all samples with an expected number of 0.1 phage genomes will contain a single phage genome, the others none.

Analysis of the amplified dilution series revealed that the detection limit was reached when samples contained an expected number between 0.1 and 2.8 phages, depending on phages and primers used. This suggests that results obtained by qPCR are congruent with those of a plaque assay.

Example: Prophage induction in single, double, and triple lysogens. Four triple lysogens (λ/HK022/mEpX1/mEp235, λ/mEp213/mEp235, λ/mEpX1/mEp235, λ/mEp/X1/mEpX2), as well as all possible single and double lysogens of these phages were constructed and validated as described previously. Of each lysogen, seven independent lysates were obtained and treated, and the amount of individual phages was quantified by qPCR. To obtain standards against which phages can be quantified, one lysate from each single lysogen was quantified with a plaque assay and used in a dilution series with five steps and two replicates each.

Productivity as measured in single lysogens is interpreted as the baseline performance of a phage. All measurements from double and triple lysogens were normalized by expressing them relative to the baseline performance of the respective phage. Results from single and double lysogens were in accordance with an earlier study, where measurements were obtained with a plaque assay. Together with the small variation among replicates, this indicates that quantification with qPCR provides reliable results (Fig. 4).

Results were analyzed by first grouping them according to each of the four triple lysogens and the corresponding double lysogens (which corresponds to the four panels in Fig. 4). Data were then log-transformed and analyzed with analysis of variance including a single factor that separated all nine titer measurements in the different lysogens (which corresponds to the nine box-plots in each panel of Fig. 4). Titers of the same phage in lysates from different lysogens were compared with Tukey’s HSD test at a 95% confidence level.

The results show that induction of double lysogens that contain phage λ together with either phage HK022, mEp213, or mEpX1, results in lysates where the titer of both phages is strongly reduced. Other double lysogens did not exhibit such a strong interference, as always one phage kept its titer close to its baseline performance. Interestingly, the negative effect of phage λ on phages HK022, mEp213, and mEpX1, was removed by the addition of either phage mEp235 or mEpX2 (Fig. 4). In these triple lysogens, the titer of phage λ was still strongly reduced, yet the titer of phages HK022, mEp213, or mEpX1 increased about a hundred-fold to a level similar to that of the corresponding double lysogen that does not contain phage λ. The results show that the interaction of multiple prophages can have unexpected outcomes that are not predictable based on observations of individual phages. The mechanism behind these interactions remains to be explained.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure 4. Quantification of phages that are released upon induction of lysogens that carry multiple prophages. Reproductive success of every phage is given relative to that achieved in a single lysogen. Box-plots summarize the results of seven replicates. Colors indicate data from phage λ (yellow), phages HK022, mEp213, or mEpX1 (blue) and phages mEp235 or mEpX2 (brown).

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