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Quantitative analysis of the bacteriophage Qβ infection cycle

Koji Tsukada [a], Misato Okazaki [a], Hiroshi Kita [b], Yoshio Inokuchi [c], Itaru Urabe [a], Tetsuya Yomo [b,d,e,*]

[a] Department of Biotechnology, Graduate School of Engineering, Osaka University, Osaka 565-0871, Japan
[b] The Complex System Biology Project, Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Osaka 565-0871, Japan
[c] Department of Bioscience, Teikyo University, Utsunomiya 320-8551, Japan
[d] Department of Bioinformatics Engineering, Graduate School of Information Science and Technology, Osaka 565-0871, Japan
[e] Graduate School of Frontier Biosciences, Osaka University, Osaka 565-0871, Japan

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ABSTRACT

In this study, the infection cycle of bacteriophage Qβ was investigated. Adsorption of bacteriophage Qβ to *Escherichia coli* is explained in terms of a collision reaction, the rate constant of which was estimated to be 4×10^{-10} ml/cells/min. In infected cells, approximately 130 molecules of β-subunit and 2×10^5 molecules of coat protein were translated in 15 min. Replication of Qβ RNA proceeded in 2 steps—an exponential phase until 20 min and a non-exponential phase after 30 min. Prior to the burst of infected cells, phage RNAs and coat proteins accumulated in the cells at an average of up to 2300 molecules and 5×10^5 molecules, respectively. An average of 90 infectious phage particles per infected cell was released during a single infection cycle up to 105 min.

1. Introduction

Viruses propagate themselves via an infection cycle. Since the basic properties of life are observed in this cycle, the study of viruses has long been an attractive field of research. Bacteriophage replication in particular has proved to be a good model system for understanding the principles of heredity, multiplication, and adaptive evolution [1–3], and research using these organisms has yielded a number of important findings [4–10].

The structure and replication of a phage genome and the molecular mechanisms of phage protein synthesis have been studied qualitatively [11]. However, the number of quantitative studies on the infection cycle has been limited. RNA bacteriophages have been extensively studied owing to their relatively short genome–which encodes a small number of proteins–and their propagation via a simple infection cycle [12]. With regard to RNA phages, our understanding is usually based on a number of studies on several types of RNA phages (e.g., MS2 phage, f2 phage, R17 phage, and Qβ phage) [12]. In order to understand the survival strategy of a phage and host-phage co-evolutionary relationship precisely, quantitative analysis of the entire infection cycle of an identical phage that is significant to theoretical biology such as phage ecology and quasispecies theory is necessary [8–10].

As a model bacteriophage, we used bacteriophage Qβ (hereafter, Qβ phage; *Allolevivirus*). This phage primarily infects gram-negative bacteria that possess a gene coding for an F or sex pilus and has a 4217-nt single-stranded RNA genome. This genome is replicated by the phage-specific enzyme Qβ replicase [13,14], which is formed by the assembly of 4 subunits. The β subunit is phage-derived; the remaining subunits, derived from the host cell, are ribosomal protein S1 [15] and the protein synthesis elongation factors EF-Tu and EF-Ts [16]. Qβ replicase has been purified [17–19], and its catalytic reactions studied extensively [20–23].

The infection cycle of the Qβ phage comprises 3 major processes, namely, adsorption on the host cell, biosynthesis of phage proteins and phage RNA in the infected cells, and progeny phage release. Eigen et al. studied the kinetics of phage RNA replication and phage protein biosynthesis in the infection cycle of Qβ phage and presented the hypercycle relationship between RNA replication and translation [24]. However, the processes of phage adsorption and progeny phage release remain unclear. In this study, we attempt to answer the following questions that are essential for understanding phage reproduction under a given physiological condition.

(1) Rate constant of phage adsorption. Qβ phage is adsorbed to the F pilus of host bacterial cell [25,26], and the RNA genome of the phage invades into the host. What are the kinetics of phage adsorption?
(2) The amounts of phage proteins translated in infected bacteria. The phage RNA invades into the host cell and functions as a template for the translation of the following phage proteins: β subunit of Qβ replicase [19,27], coat proteins that are the building blocks of a phage particle, maturation A_2 protein that has a lytic function [12], and maturation A_1 protein (readthrough protein) [12]. What amounts of phage proteins

* Corresponding author. Department of Bioinformatics Engineering, Graduate School of Information Science and Technology, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 4171; fax: +81 6 6879 7428.
E-mail address: yomo@ist.osaka-u.ac.jp (T. Yomo).

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are produced in infected cells? In the case of Qβ replicase, Horiuchi and Matsuhashi reported a change in the enzyme activity in the crude extract of phage-infected cells [28]; however, the actual amount of the β subunit of Qβ replicase produced in a phage infection cycle has not been reported.

(3) The amount of phage RNA replicated in infected bacteria. What are the amount of RNA genome and kinetics of its replication in infected cells? Is there any correlation between the amount of phage RNA and phage proteins?

(4) Time distribution of the burst of infected bacteria and the number of phage particles released per infected cell. At the final step of a phage infection cycle, progeny phages are released into the culture medium. At what time do infected cells burst after phage adsorption? How many new phage particles are generated by an infected cell?

In the present study, we analyzed part of the infection cycle of Qβ phage and present a quantitative description of the basic processes of phage adsorption, phage RNA and protein synthesis, and cell lysis following prophage release. We first reported the rate constant of Qβ phage adsorption and concentration of the β subunit of Qβ replicase. Both the phage coat protein and RNA genome were overproduced with respect to the number of infectious progeny phages released in the culture medium under the studied physiological condition. This quantitative information will make a significant contribution to our understanding of the mechanism of RNA bacteriophage proliferation, population of RNA phage quasispecies [9], and evolvability of RNA phage.

2. Materials and methods

2.1. Bacterial strains, plasmids, and antibodies

Escherichia coli (*E. coli*) strain K-12 F^+ (A/λ) (*sup, pro/*F^+) was used for all infection experiments and DH5αPRO (Clonetech, Mountain View, CA), [*deoR*, *endA1*, *gyrA96*, *hsdR17(*rk^-mk^+*)*, *recA1*, *re/A1*, *supE44*, *thi-1*, *Δ(lacZYA-argF) U169*, *Φ80δlacZΔM15*, F^-, λ^-, P_{N25}/*tetR*, and P^q_{laci}/*laci*, Sp^r] was used for the preparation of Qβ phage as described below. All bacteria were cultured in Luria-Bertani (LB) medium (Sigma-Aldrich, St Louis, MO). A plasmid harboring a cDNA of the Qβ phage genome, pACYCQβ, was constructed by subcloning Qβ cDNA into a commercial expression vector, pACYC177. The monoclonal antibodies for the β subunit of Qβ replicase and coat protein were obtained from Monoclotech Inc. (Kobe, Japan). These antibodies were raised against the purified Qβ replicase [18] and purified coat protein fused with a hexahistidine tag at the C-terminus, respectively. Anti-mouse IgG HRP conjugate was purchased from Promega (Madison, WI). All chemicals used in this study were of the highest grade commercially available.

2.2. Preparation of Qβ phage

It is known that Qβ phage can be harvested from bacteria transformed with a Qβ DNA-containing plasmid [29]. In order to obtain the wild-type Qβ phage routinely, we transformed DH5αPRO cells with pACYCQβ and cultured these cells in the presence of 50 μg/ml of kanamycin sulfate at 37 °C. In the culture medium, we found approximately 10^7 PFU/ml of free Qβ phage. In order to propagate the infectious Qβ phage, A/λ cells in their log-phase growth (200 ml) were mixed with the Qβ phage harvested from the DH5αPRO/pACYCQβ culture. The infection was initiated in the presence of 10 mM $CaCl_2$ at a multiplicity of infection (MOI) of 0.01. After incubation at 37 °C for 5 h, chloroform was added to the infection mixture and this was incubated for a further 30 min. Intact bacterial cells and cell debris were both precipitated by centrifugation at 5500 ×*g* for 20 min at 4 °C (Hitachi CR-22; Hitachi, Japan), and the supernatant was decontaminated using a sterile cellulose nitrate membrane filter (pore size, 0.22 μm; Corning, NY). Approximately 10^{10} PFU/ml of phage was obtained in the supernatant (approximately 200 ml). In order to precipitate the phage particles, polyethylene glycol (PEG) solution [30% (v/v) PEG 6000 in 2.5 M NaCl] was added to the filtered phage solution to yield a final PEG concentration of 9% [30]. After allowing to stand for 1 h on ice, free phage particles were precipitated by centrifugation at 15,000 ×*g* for 30 min. Ice-cold P buffer [50 mM Tris–HCl (pH 7.6), 0.1 M NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA•2Na] was added to the precipitate to suspend the phage. The phage suspension (approximately 5 ml) was dialyzed twice against 1 l of P buffer at 4 °C for 24 h using a Slide-A-Lyzer (10K MWCO; Pierce Biotechnology, Rockford, IL). The dialyzed phage solution was filtered (pore size, 0.22 μm; Millipore, Billerica, MA) and glycerol to a final concentration of 40% was added. The phage solution was stored at −20 °C and subsequently used for the infection experiments. The plaque-forming activity of the solution (approximately 10^{11} PFU/ml) did not change for more than a year.

2.3. Estimation of the number of living bacteria and infectious phage particles in culture medium

In order to measure the number of living *E. coli* cells in the culture medium, we multiplied the value of colony-forming units (CFU) by 2.9 (see Results). For CFU counting, the bacterial culture medium was diluted with fresh LB medium to an appropriate bacterial cell density, and then 100 μl of the diluted culture was inoculated onto an LB agar plate. The bacteria were cultured for more than 15 h at 37 °C, and the resulting colony number was counted. Further, in order to estimate the number of infectious Qβ phage particles in the culture medium, we multiplied the value of plaque-forming units (PFU), obtained by the standard procedure [31], by 1.9 (see Results).

2.4. Phage adsorption

E. coli strain A/λ was precipitated by centrifugation and resuspended in fresh LB medium containing 10 mM $CaCl_2$ to a density of 10^7–10^9 cells/ml. Since the F pilus of *E. coli* is known to be destructible, it will be destroyed by centrifugation and resuspension with vortex mixing. Novotny et al. reported in 1969 that F pilus, which is important for male-specific bacteriophage adsorption, can reappear in rich culture medium [32]. In order to determine the appropriate preincubation time before the challenge of Qβ phage, we tested the adsorption of Qβ phage to bacterial cells at different preincubation times. Although there was no difference in the rate constant of adsorption between 30 and 60 min, an approximately 10 times higher adsorption rate constant was estimated for the cells incubated for more than 30 min. Based on this observation, we preincubated cells at 37 °C for 30 min before mixing with various concentrations of Qβ phage. During the incubation, unadsorbed phage particles were separated using a sterile syringe-driven polyvinylidene difluoride (PVDF) membrane filter unit (pore size, 0.22 μm; Millipore). The phage particle concentration in each filtered sample was measured as stated above.

2.5. Production of phage proteins in phage-adsorbed bacteria

E. coli strain A/λ was precipitated by centrifugation and resuspended in fresh LB medium (100 ml) containing 10 mM $CaCl_2$. After resuspension, the cells were preincubated at 37 °C for 30 min without shaking and Qβ phage (2×10^9 particles/ml) were added to the *E. coli* A/λ cells (6×10^8 cells/ml). A 10-ml aliquot of the mixture was drawn every 15 min for 45 min and immediately chilled on ice. Bacterial cells were collected by centrifugation at 5000 ×*g* for 10 min at 4 °C and stored at −80 °C until use. Proteins extracted from frozen cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a PVDF membrane (Promega, Madison, WI), and then subjected to immunoblot analysis using an antibody raised

against the β subunit or coat protein. Protein bands that cross-reacted with the antibody were detected using horseradish peroxidase-conjugated second antibody and ECL Advance Western blotting detection reagents (GE Healthcare UK Ltd., England) according to the manufacturer's instructions. The amount of both β subunit and coat protein on the gel was quantified using the purified proteins of known concentration.

2.6. Qβ RNA isolation from infected cells and quantification

E. coli strain A/λ was precipitated by centrifugation and resuspended in fresh LB medium (10 ml) containing 10 mM $CaCl_2$ and the cells were preincubated for 30 min at 37 °C in a standing culture. A final concentration of 2×10^7 particles/ml of Qβ phage was added to the *E. coli* A/λ cells (3×10^8 cells/ml). A 1-ml aliquot of the mixture was drawn every 5 min after phage addition and immediately chilled on ice. The bacterial cells were collected by centrifugation at 5500 ×*g* for 10 min at 4 °C and stored at −80 °C until use. Total RNA was extracted from frozen cells using a Qiagen RNeasy® mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The amount of phage RNA was measured by quantitative real-time polymerase chain reaction (PCR). Reverse transcription was performed as described below. The RNA samples diluted 10,000-fold with 1 mM EDTA were heated at 95 °C for 5 min to denature double-stranded regions and mixed with reverse transcriptase (Prime Script RTase; Takara Bio Inc., Ohtsu, Japan) and the primer 5'-TAAGCGAATGTTGCGAGCACCTTGTATGGTCCGTAATCAC-3'. Reactions were performed according to the manufacturer's instructions. The cDNA samples were then diluted 100-fold with water and mixed with qPCR MasterMix (Eurogentec, Liege, Belgium), a dual-labeled probe, 5'-FAM-TGCCCTCGTCGGATCGGTCCTAAT-BHQ-1-3' (Sigma-Aldrich, St Louis, MO), and the primers 5'-TAAGCGAATGTTGCGAGCAC-3' and 5'-TGCCTAAACAGCTGCAACGT-3'. Reactions were carried out using an Mx3005P™ QPCR System (Stratagene, La Jolla, CA). The amount of RNA was quantified using a standard curve prepared from known concentrations of phage RNA transcribed *in vitro*; the concentrations were determined by absorbance at 260 nm.

3. Results

3.1. Measurement of the number of living bacteria and infectious phage in a culture medium

For quantitative analysis of the Qβ phage infection cycle, measurement of the number of bacterial cells and phage particles in infection experiments is crucial. Bacterial cell number is generally estimated by a colony-counting method and phage number is determined by a plaque-counting method. For colony counting, bacterial cells are inoculated onto an agar plate and cultured until visible colonies are observed; for plaque counting, phage particles are diluted and mixed with a large number of phage-sensitive bacterial cells, and the mixture is cultured in a layer of soft agar until plaques are observed. Since phage infection experiments were performed in a quiescent liquid culture, we used an alternative method for counting bacterial cells and phage particles in order to assess the differences in estimates of the number of living bacteria and infectious phage particles.

For measuring the number of living bacteria under quiescent liquid culture conditions, we inoculated A/λ cells at an average of 0.15 CFU each (100 μl) into 48 wells and incubated these for 5 h at 37 °C. We observed more than 1000 CFU in 17 of the 48 wells and no colonies in the remaining 31 wells. If the average number of living bacteria per well is n, then the fraction of the wells, p_0, in which no bacterial growth was detected is estimated by the Poisson law.

$$p_0 = e^{-n} \tag{1}$$

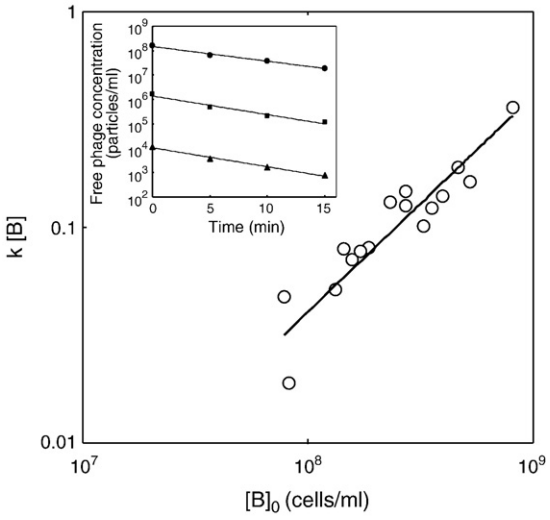

Fig. 1. The adsorption of Qβ phage to bacteria. Concentrations of free phage were analyzed for 15 min after mixing phage and bacteria. When bacterial solutions at the same concentration (approximately 10^9 cells/ml) were mixed with 10^8 (●), 10^6 (■), and 10^4 (▲) particles/ml of infectious phage, the decreases in free phage concentration could be fitted to *pseudo*-first-order kinetics (inset). The slopes, indicated by $k[B]$, were estimated and plotted against the initial density of bacterial cells, $[B]_0$. The rate constant for Qβ phage adsorption to bacteria, k, was estimated to be 4×10^{-10} ml/cells/min from the slope of a linear regression line.

In the present study, p_0 was 31/48 and n was estimated to be 0.44. The value 0.44 is 2.9 times 0.15—the average cell value for each well estimated by colony counting. Therefore, in order to estimate the number of living *E. coli* cells in the culture medium, we multiplied the CFU value by 2.9.

For measuring the number of infectious Qβ phage particles in the quiescent liquid culture, we used a single-burst experiment [33]. When a bacterial culture (more than 10^8 CFU/ml) was mixed with Qβ phage for 15 min at an MOI of 0.01–0.1, each Qβ phage particle was assumed to adsorb to a bacterial cell (Fig. 1). Therefore, the phage-adsorbed cell number after 15 min was used to estimate the number of phage particles added. The phage-adsorbed cells were diluted to an average density of 0.08, 0.25, 0.5, and 1.0 PFU/well and 100 μl of each diluted culture was dispensed equally into microwells (48 or 96 wells). After a 2-h culture at 37 °C, the entire contents of each well were plated for plaque counting. During the 2-h incubation, all phage-adsorbed bacteria burst and many progeny phage were released (Fig. 2). We observed multiple plaques when wells received a phage-adsorbed cell. When wells did not receive phage-infected cells ("vacant wells"), one or no plaque was detected. By measuring the fraction of vacant wells, p_0 was given by Eq. (1). In our experiments, the n values 0.17, 0.50, 0.88, and 1.9 were estimated from 0.08, 0.25, 0.5, and 1.0 PFU/well, respectively. Since the n value was on average 1.9 times larger than the PFU value, in order to estimate the number of infectious phage particles in the culture medium, we multiplied the PFU value by 1.9.

3.2. Rate constant of Qβ phage adsorption to the host bacteria

The first step in the phage infection cycle is adsorption. The processes by which various phages adsorb to their host cells have been analyzed since the 1930s [26,33–39]. The phage adsorption rate is expressed as follows:

$$-\frac{d[P]}{dt} = k[B][P], \tag{2}$$

where k (ml/cells/min) is the adsorption rate constant, $[B]$ is the bacterial cell concentration (cells/ml), and $[P]$ is the unadsorbed phage concentration (particles/ml) [33,39,40].

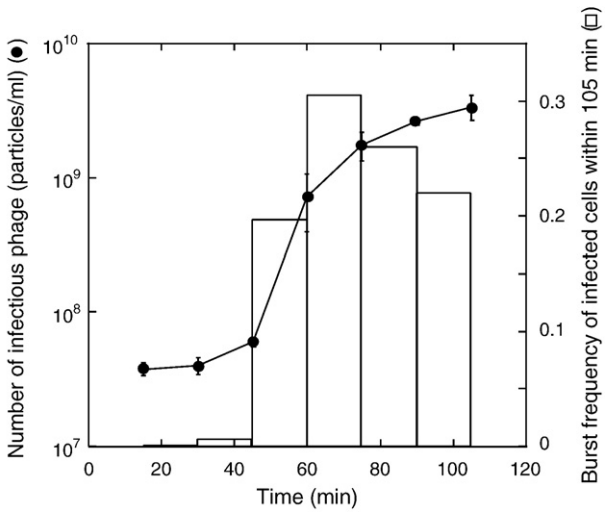

Fig. 2. One-step growth of Qβ phage. Qβ phage (approximately 1.9×10^7 particles/ml) were added to a bacterial culture (2.9×10^8 cells/ml). After 15 min of adsorption, the infection mixture was diluted 10^4-fold with LB medium without addition of calcium ion (calcium ion is needed for phage adsorption) so that the rate of phage adsorption was slowed down by more than 10^{-8} fold. Thus, any further adsorption of phage particles to bacterial cells was neglected during further incubation. As the incubation time progressed, the plaque number (●) increased due to the release of free phage particles from infected cells. As the burst of the infected bacteria occurred, the plaque number increased due to the release of free phage particles from infected bacteria. The average number of phage particles per infected cell up to 105 min was estimated to be 90. Data are expressed as the mean±SD ($n=3$). The time distribution of burst frequency of infected cells is also represented as a histogram on the assumption that every infected cell released an average of 90 progeny phage up to 105 min.

Qβ phage adsorbs specifically on the F pilus. The decrease in the rate of free Qβ phage particles in cultures was measured in every infection experiment at various concentrations of phage particles and bacteria. In order to prevent complexity due to multiple infections, we added phage particles to the bacterial cell culture (10^8–10^9 cells/ml) under bacterial excess. When 9×10^8 cells/ml bacteria (in excess to the phage concentration) was mixed with phage at approximately 10^8, 10^6, or 10^4 particles/ml, the time course of free phage decrease appeared to follow the kinetics of a *pseudo*-first-order reaction (Fig. 1, inset). When we used various concentrations of bacteria, the same free phage decrease kinetics was observed in each infection experiment. Therefore, the adsorption rate of Qβ phage can be given as Eq. (2). Since the slopes of the regression lines ($k[B]$) did not change between 0 and 15 min, we can substitute the initial bacterial cell concentration ($[B]_0$) for $[B]$. The results indicated that the dissociation of phage particles can be neglected within 15 min. In order to obtain the rate constant value, k, phage adsorption experiments were conducted with various bacterial cell concentrations ($[B]_0$). In every adsorption experiment, values of $k[B]_0$ were obtained and plotted against $[B]_0$ (Fig. 1). Linear correlation was found and a k value of 4×10^{-10} ml/cells/min ($R^2=0.88$) was estimated from the regression line slope.

3.3. Average number of progeny phage released from an infected cell and its time distribution

In order to investigate the time course of free phage release and average burst size, we conducted one-step growth experiments [33,40,41], as described in the legend to Fig. 2. The average multiplication of phage particles was estimated to be approximately 90 from the phage increase between 15 and 105 min (Fig. 2). This value was considered as the lower limit of the average number of progeny phage released from the burst of an infected cell because the free phage number continued to increase at 105 min. Assuming that every infected cell burst releases 90 infectious phage, the burst frequency every 15 min until 105 min was estimated (Fig. 2, histogram).

The burst frequency was low until 45 min after phage adsorption. The maximum burst frequency was observed between 60 and 75 min. Although most phages adsorbed to bacterial cells in 15 min, the release of progeny phage was observed over a period of 1 h from 45 to 105 min.

3.4. Production of phage proteins and replication of phage genome RNA in phage-infected bacteria

Most bacterial cells to which an infectious phage adsorbed started bursting more than 45 min after phage adsorption (Fig. 2). In order to analyze the early processes of infection dynamics, we measured the average amounts of phage proteins and RNA genome produced in an infected cell between 15 to 45 min after phage adsorption (Fig. 3).

Approximately 130 molecules of the β-subunit of Qβ replicase had been translated by 15 min and approximately 450 molecules had continuously accumulated by 45 min (Fig. 3, closed circles). For coat proteins, approximately 2×10^5 molecules had been translated by 15 min and approximately 5×10^5 molecules had accumulated by 45 min (Fig. 3, closed squares). A phage particle comprises 176 coat proteins [42]—more than 1000 particle-equivalent coat proteins were translated in 15 min and approximately 2900 particle-equivalent coat proteins had accumulated by 45 min.

In infected cells, phage RNA is replicated by Qβ replicase [14]. Since real-time PCR using the reverse transcriptase reaction is suitable for quantitative estimates of phage RNA [43–45], we measured the amount of phage RNA(+) replicated in phage-adsorbed cells using a strand-specific reverse transcriptase reaction combined with quantitative PCR (Fig. 3, open triangles). Whereas the average concentration of Qβ RNA(+) in infected cells was below the limits of detection until 10 min (12 molecules per cell), 18 molecules had been replicated by 15 min. Since the β subunit number (130 molecules) was approximately 7 times that of Qβ RNA(+) at 15 min, RNA replication was considered to be exponential [20], and we estimated its first-order rate constant at 15 min to be approximately

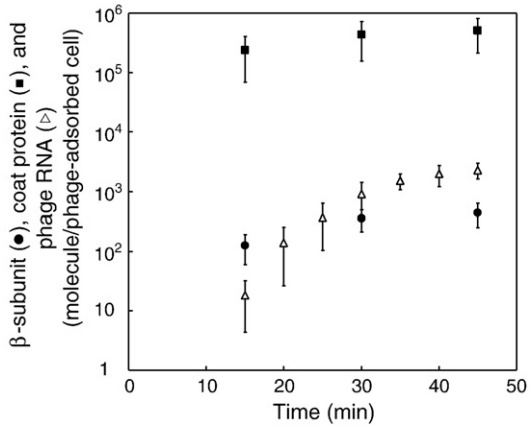

Fig. 3. Average infection cycle of Qβ phage in phage-adsorbed bacteria. The number of β-subunits (●), coat proteins (■), and genomic RNA of Qβ phage (Δ) was measured and the average number of molecules per infected cell was calculated. The number of phage proteins was measured by mixing bacterial cells with high phage concentration. After 15 min, all bacterial cells were considered to have undergone phage adsorption. The number of phage RNA(+) was measured by infecting bacteria with a low concentration of phage. We were able to estimate the number of phage-adsorbed cells by Eq. (2) with assignment of values as follows: $[B]$ as the initial concentration of bacterial cells, $[P]$ as the initial concentration of phage particles, and the adsorption rate constant $k=4\times10^{-10}$ ml/cells/min.

0.005/s. Approximately 2300 phage RNAs(+) had accumulated by 45 min.

4. Discussion

The survival strategy of bacteriophage encompasses the key mechanisms of life. Therefore, quantitative analysis of the whole infection cycle is important. Among all phages, Qβ phage is the most suitable for such analysis since it has a small RNA genome and the structure and kinetics of the specific enzyme for Qβ RNA replication (Qβ replicase) have been well studied.

Eigen et al. [24] measured the rates of protein biosynthesis and phage RNA replication as well as the amount of progeny phage produced in the infection cycle of Qβ phage and reported a hypercycle–coupling of phage RNA and protein biosynthesis–with kinetic considerations. They treated the phage-infected bacteria with toluene to transport [α-^{32}P]-UTP or [U-^{14}C]-Leu across the membrane into the bacterial cells in order to measure the rate of RNA replication and protein synthesis in the infected cells. Not only they successfully documented that the rate of RNA replication was accelerated by the hypercycle but also reported the rates of RNA replication and coat protein translation (about 30 min after phage infection), which were 300 molecules/min and 30,000 molecules/min, respectively. In our study, RNA replication rate was estimated to be approximately 100 molecules/min from 30 to 45 min and the rate of coat protein translation was approximately 1×10^4 molecules/min (Fig. 3). Therefore, the rates of RNA replication and phage protein translation in the toluene-treated phage-infected bacteria were 3 times higher than those in our bacteria infected by the phage under physiological conditions. Because toluene-treated bacteria escaped lysis, Eigen et al. [24] did not observe the progeny phage release under physiological conditions. Thus, the following processes of the infection cycle were not described: the process of phage adsorption, amount of β subunit produced, relationship between the amount of β subunit produced and phage RNA replicated in the infected cells, and time course of the burst of the infected cells.

In this study, we analyzed part of the infection cycle and described the multiplication process of Qβ phage quantitatively. For the analysis under physiological conditions, we took great precautions in the preparation of both Qβ phage and bacterial host—each was prepared stably and reproducibly.

Further, we found that the colony count was approximately one-third the number of bacterial cells and that the plaque count was approximately one-half the number of phage particles.

We estimated the ratio between the plaque count and the number of Qβ phage particles as approximately 0.53 (1/1.9); this value is consistent with that reported for T-even phages [46].

The adsorption of Qβ phage particles to bacteria can be expressed as a collision reaction similar to that in other phages. The second-order rate constant for Qβ phage adsorption was measured as $k=4\times10^{-10}$ ml/cells/min (equivalent to approximately 1.1×10^{-9} ml/CFU/min; Fig. 1). The adsorption process of phages has been studied extensively and the rate constants of various phages have been previously reported [3,36,39,47].

It is interesting to note that the adsorption rate constants for various phages were estimated to have almost the same order values under standard culture conditions for bacterial cells although the mechanism for phage adsorption or the number of adsorption sites were different. For example, the rate constants for the adsorption of T4 phage and Qβ phage had similar values, although T4 phage adsorbs on the surface of bacterial cells, whereas Qβ phage adsorbs on the F pilus. As consequence of the arms race between phage and bacteria, an appropriate adsorption affinity for their survival might evolve in each pair of host bacteria and phage. Compared to other phages such as R17 phage, smaller amounts of Qβ phage particles adsorb to the F pili of bacterial cells [25]; however, the rate constant of Qβ phage adsorption was found to be almost equal to that of other phages.

How did the replication of phage RNA(+) in the infected bacterial cells proceed? Since the number of translated β subunits was almost 10 times higher than that of Qβ RNA(+) (Fig. 3) at 15 min, the replication of Qβ RNA(+) was catalyzed in an exponential manner at around 15 min. Therefore, a first-order reaction of RNA replication was considered and its rate constant was estimated to be 0.005/s from the RNA(+) concentrations at both 15 and 20 min. This rate constant is similar to that of RNA replication in exponential phase *in vitro* (0.006/s) [20].

Although the number of β subunits increased from 300 to 450 molecules/cell between 30 and 45 min, there was no acceleration in the rate of RNA replication (Fig. 3). There could be several reasons for this observation, 2 of which are as follows. First, due to the insolubility of the β-subunit [21], additional translation of the β subunit could cause an aggregation after 30 min and there could be a limitation to the accumulation of active Qβ replicase. Second, the half-life of Qβ replicase *in vivo* may be so short that the RNA replication reaction could be catalyzed by freshly synthesized enzymes.

After phage adsorption, invaded Qβ RNA(+) functions as an mRNA for translation of the phage proteins, but most replicated RNA(+) in infected cells appeared to be unfunctional. Phage RNA(+) increased up to 150-fold in phage-adsorbed cells between 15 and 45 min, whereas there was no acceleration in the translation of phage proteins (Fig. 3). This implies that most replicated phage RNAs(+) did not function as a template for translation of both coat protein and β subunit. A possible explanation for this observation is that the translation of phage proteins is inhibited by the binding of a repressor. There was an approximate 1000 times difference between the amount of β subunits and coat proteins (Fig. 3). Coat protein is a feasible candidate for a translation repressor since it has been demonstrated that this protein can bind to the phage genome and serve as a repressor of β subunit translation by binding to the Shine–Dalgano sequence of the β subunit gene [48–50]. However, it is not clear whether a coat protein binding to the phage genome can inhibit its own translation under physiological conditions or whether the rate of coat protein translation in infected cells would be extremely high for further acceleration. Another possibility is that most nascent RNA(+) is encapsulated into a phage particle before ribosomal binding. Further studies on the intracellular dynamics of phage production, the number of minus strands of the Qβ RNA genome, the amount of the other subunits of Qβ replicase, the time course of the encapsulation of the phage RNA genome, and the change in translation rate of host protein would be useful.

In summary, we analyzed the infection cycle of Qβ phage under physiological conditions. The mode of Qβ phage adsorption was expressed as a collision reaction and its second-order rate constant k was estimated as 4×10^{-10} ml/cells/min. It was found that after phage adsorption, the β subunit of Qβ replicase accumulates in the infected cells and that the replication of the phage genome *in vivo* proceeds in a manner similar to that *in vitro*. The amount of coat protein reached approximately 2900 particle equivalents per infected cell and Qβ RNA (+) had accumulated to approximately 2300 molecules/cell after 45 min. Further, an average of 90 phage particles per infected cell had been released by 105 min. The time distribution of progeny phage release was over 1 h. Therefore, the average number of released progeny phage was only 3–4% of the numbers of coat proteins or phage RNA that had accumulated in the infected cells by 45 min. According to the study by Eigen et al. [24], the number of intracellular phages was 600 infective phage particles per infected cell, which corresponded to approximately 10,000 phage particles, by artificial digestion of the cells with lysozyme. Therefore, we believe that the overproduction of the Qβ phage proteins and RNA does not depend on the experimental conditions or bacterial strains. It is interesting to assess whether the overproduction of phage proteins and RNA plays an important role in the survival of the infectious Qβ phage. In order to further elucidate the basic mechanisms of phage multiplication,

quantitative studies on processes such as the time course and production of maturation A_2 protein, known as lytic protein [51,52], in infected cells and the efficiency of packing of the phage RNA into a phage particle need to be conducted. In order to understand bacteriophage infection cycle with regard to theoretical biology or mathematics, quantitative information obtained from careful analyses is essentially required. Our study will contribute to the thorough understanding of the key mechanism underlying RNA bacteriophage proliferation and validation of the quasispecies theory in RNA phage life cycle [9] as a model experimental system.

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