RESEARCH LETTER

# Impact of the cell life-cycle on bacteriophage T4 infection

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## Abstract

Synchronized *Escherichia coli* cultures were infected with bacteriophage T4 at discrete points in the cell growth cycle. The cell cycle had a significant impact on the outcome of infection. Cell burst size was smallest for newly formed cells and increased dramatically as these progressed in the cell cycle. The largest burst sizes were achieved when infecting cells immediately prior to cell division. When cells were infected during cell division, the burst size was reduced back to its initial value. Interestingly, lysis time was longest for young cells, reached a minimum at the same point that burst size reached its maximum value, and then increased at the commencement of cell division. Consequently, phage productivity in cells about to undergo cell division was almost three times greater than the productivity of young, newly formed cells. The availability of intracellular resources is believed to be the major driving force behind phage productivity during infection. Indeed, intracellular RNA contents at the time of infection were found to correlate strongly with phage productivity. There was no significant relationship between cell DNA levels and phage productivity. Finally, burst size experiments suggested that the cell cycle also influenced the likelihood of a phage to undergo productive infection.

## Introduction

The state of the host cell during bacteriophage DNA penetration will impact the trajectory of the infection. In addition to the well-studied growth associated parameters such as specific growth rate, temperature, media composition, and ion concentration (Adams, 1959; Kutter *et al.*, 1994; Hadas *et al.*, 1997), cell size, age, and composition can also dictate the fecundity of a bacteriophage infection (Hadas *et al.*, 1997; Rabinovitch *et al.*, 1999; You *et al.*, 2002).

A study on the development of phage T7 found that phage infections became increasingly productive as the growth rate of the host increased, mainly due to the higher concentration and elongation rate of ribosomes at higher host cell growth rates (You *et al.*, 2002). The concentrations of genomic DNA, amino acids, and host cell RNA polymerases also affected phage growth, although to a lesser degree. At least two studies on phage T4 have found evidence that burst size of the cell may increase if the cell is infected later in its division cycle (Hadas *et al.*, 1997; Rabinovitch *et al.*, 1999). The authors hypothesized

that the larger cellular protein synthesizing system of older cells, not simply a larger cell volume, is the more important determinant of infection outcome. In addition, a study on the use of synchronized cultures of *Escherichia coli* in bacteriophage production found evidence that phage productivity was considerably larger for a synchronized culture than an asynchronous one, even at slower growth rates (Sauvageau & Cooper, 2010).

Intracellular resources may also influence the lytic/lysogeny decision in phage λ: an approximately twofold increase in cell volume at the time of a phage λ infection was reported to raise the probability of lytic growth (St-Pierre & Endy, 2008). We report a similar phenomenon for lytic phage T4 infections and hypothesize that this correlation is due to a selective pressure to lyse the largest cells in a population.

The present study demonstrates the specific influence of the cell cycle on a phage T4 infection. Using synchronized cultures, it was possible to get snapshots of phage T4 infections initiated at discrete points in the *E. coli* cell cycle. Synchronized cultures were generated using a cyclical culturing technique called self-cycling fermentation

(SCF), which maintains bacterial cultures in synchronized, logarithmic growth (Sheppard & Cooper, 1990; Brown & Cooper, 1991; Brown, 2001). The SCF synchronization strategy has many marked advantages over other common synchronization methods such as Helmstetter's 'baby machine' (Helmstetter & Cummings, 1963) or continuous phasing (Dawson, 1972). The cell densities obtained are comparable to those of a batch, it can easily be set-up to run as a semi-continuous, fully automated process using non-intrusive instrumentation, a growth-associated parameter determines the conditions for cycling, and synchrony indices upwards of 0.6 can be readily achieved.

One of the signature properties of a synchronized culture is a narrow distribution of cell ages, leading the culture to display the characteristics of an individual cell rather than that of an average cell. Consequently, phage T4 infections of a culture initiated at different points during synchronized growth can serve as a measurement for the infection of a single cell at different stages in its cell cycle. In this way, this study reports, for the first time, the impact of the cell cycle on bacteriophage production during lytic infection.

## Materials and methods

### Microorganisms, medium and reactor operation

The microorganisms used in this study were *E. coli* ATCC 11303™ and bacteriophage T4 ATCC 11303-B4™. The bacteria was cultured and infected in a minimal salt medium (MSM) at 37 °C containing 3 g L$^{-1}$ D-glucose and 0.025 g L$^{-1}$ yeast extract, as previously described (Sauvageau *et al.*, 2010). Cultures with a synchrony index > 0.6 were generated using SCF according to a reactor set-up and operating conditions previously described (Sauvageau *et al.*, 2010). Viable cell counts were performed using standard plate assays. For further information regarding quantification of cell synchrony, see Blumenthal & Zahler (1962).

### Burst size and lysis time measurements

Phage burst size (*b*) was determined using a standard protocol modified from one-step growth methods reported in the literature (Ellis & Delbrück, 1939; Wang, 2006). A 1-mL sample of *E. coli* (*c.* 10$^8$ cells mL$^{-1}$) was mixed with phage T4 at a multiplicity of infection (MOI) of 0.1. After incubation for 5 min at 37 °C, a 10-μL aliquot was diluted 10 000-fold in fresh media. A 1-mL sample was removed and assayed to obtain the total infective centers ($T_0$, free floating phages plus bacteria infected with phage). The original infection broth was

vortexed with 2% chloroform to disrupt host cells and then assayed to determine the unadsorbed phage concentration (*U*). The diluted culture was incubated in a 250-mL shake flask at 37 °C and 220 r.p.m. for 35 min, then diluted a further 10 times. At 50 min, three 1-mL samples were removed and assayed for final total phage ($T_F$). Burst size was then calculated as $b = T_F/(T_0-U)$.

Lysis time was determined by closely following the evolution of a phage T4 infection using spectrophotometry at a wavelength of 600 nm. A 1-mL sample of *E. coli* culture (*c.* 10$^8$ cells mL$^{-1}$) was mixed in a sterile 3-mL disposable cuvette with a pre-warmed 1-mL aliquot of phage T4 at an MOI of *c.* 1.5. The mixture was incubated at 37 °C for 2 min before 1 mL of media was added and agitation began at 220 r.p.m. Optical density was monitored every 5 min for 20 min, and every 2 min thereafter until cell lysis was identified as a significant drop in the culture optical density. Here the lysis time corresponded to the time at which the peak in optical density occurred; this point representing the onset of lysis. The burst size and lysis time data was used to calculate the phage productivity during infection.

### Isolation and analysis of RNA/DNA

Samples of *E. coli* cultures were collected during synchronized logarithmic growth in 0.5-mL aliquots, centrifuged at 14 000 *g* for 1 min, and stored at −80 °C. An RNA/DNA purification kit from Norgen Biotek Corp (Ottawa, ON, Canada) was used according to the manufacturer's instructions to isolate genomic DNA and total RNA from each sample.

Genomic DNA samples were analyzed by agarose gel electrophoresis according to established protocols (Sambrook *et al.*, 1989). Densitometry was used for quantification of the DNA concentration: band intensities of the genomic DNA in each sample were compared with the intensity of a capsid DNA standard using the open-source software IMAGEJ.

Total RNA concentration and purity were determined by measuring the absorbance of light in each sample at 260 and 280 nm using a UV-visible spectrophotometer (Genova Jenway, Staffordshire, UK). The RNA and DNA data is reported as a fraction of the cell dry weight (mass %). Dry weight was determined by measuring the optical density of the culture at 600 nm using a spectrophotometer (Varian, Cary Bio-50). A cell culture at an OD$_{600\ nm}$ of 1.0 had a dry weight of 0.5 g L$^{-1}$.

### Statistical testing

Linear regression was performed on the burst size and lysis time measurements with respect to specific RNA

concentration. The significance of the slope was tested using the *F*-statistic ($H_o$: $\beta_1 = 0$). All tests were performed at a 95% confidence interval.

## Results

Samples of synchronized cultures were removed periodically from a SCF cycle corresponding to different points in the cell life cycle (Sauvageau *et al.*, 2010), and infected with bacteriophage to determine cell burst size and lysis time. Plotting these data against the average cell age of the culture displays the growth characteristics of the cells and the influence the cell cycle has on phage infection (Fig. 1). The cell age has been normalized such that 0 represents recently divided cells beginning their growth cycle and 1 indicates that all cells have completed their growth cycle (interdivision cycle + binary fission). The cell age was normalized to account for small cycle-to-cycle variability during SCF operation (see Supporting Information, Fig. S1). Cell density has also been normalized to facilitate comparison between experiments. (For each experiment the cell density was divided by the final cell concentration.) The shaded area represents where cell division took place, characterized by a rapid increase in cell density (Fig. 1a). Synchrony is never perfect in a real system. Consequently, cell division within the culture took place in a finite time (*c.* 40% of the SCF cycle).

The burst size was smallest early in the cell cycle when the cells where youngest, having just completed cell division (Fig. 1b). Cells infected at the onset of cell division had the largest burst size. As division took place, the burst size began to decrease. The lysis time of the phage, defined here as the time between onset of infection (insertion of DNA) and onset of cell lysis, follows a complementary trend to the burst size (Fig. 1b). The longest lysis time was observed when infecting cells early in the cell cycle. It then decreased to a minimum for mature cells just as cell division got underway. Once cell division began, the lysis time increased to values equal to those seen when infecting cells at the beginning of the cell cycle. As shown in Fig. 1c, the combination of increased burst size and shortened lysis time led to a maximum phage productivity of 112 PFU cell$^{-1}$ h$^{-1}$ for cells infected immediately prior to cell division. This is almost three times greater than the productivity of cells infected early in the cell cycle (42 PFU cell$^{-1}$ h).

Analysis of intracellular DNA and RNA at the time of infection showed that burst size, lysis time, and phage productivity correlated strongly with intracellular RNA (Fig. 2a–c). Intracellular DNA was not linearly correlated with phage productivity (Fig. 2d, $P > 0.05$).

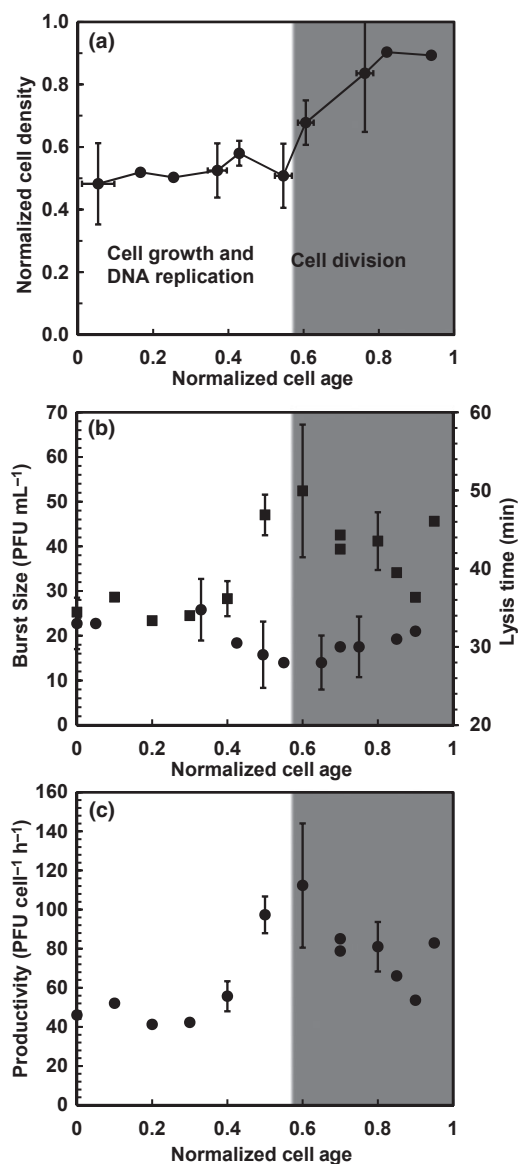Another observation from the study was that the number of successful phage infections varied throughout the



**Fig. 1.** Cell growth and phage development. (a) The cell density during one generation of a synchronized culture. One-step growth experiments using phage T4 were performed at different points in the culture generation in order to determine (b) the burst size (■) and lysis time (●), and (c) phage productivity. Error bars represent the standard deviation of at least three separate experiments.

cell cycle. The burst size experiments shown in Fig. 1 were all carried out using 10-µL aliquots from a single phage stock solution. Therefore, the measured value of the initial total infective centers (unadsorbed phage + infected host cells) would be expected to remain constant from one experiment to the next. This was not the case. Figure 3 compares the total infective centers measured 5 min post-infection for both synchronized cultures and non-synchronized (batch) cultures growing
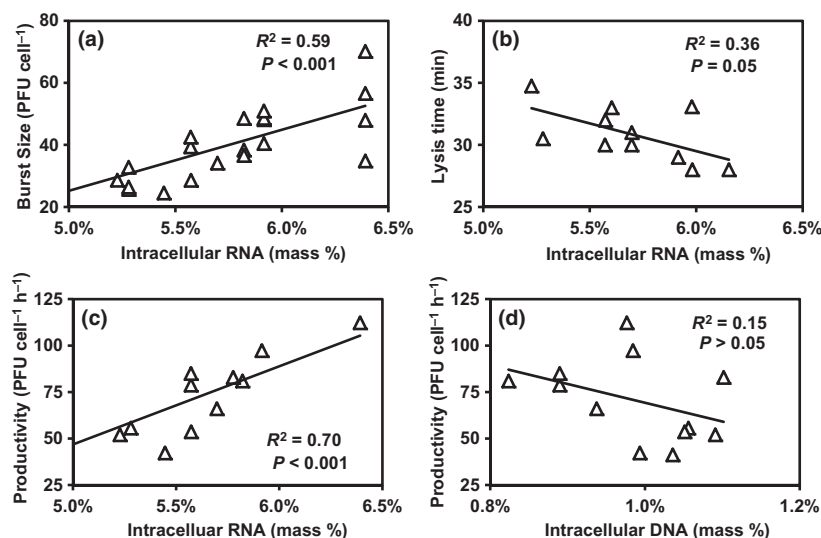
**Fig. 2.** Intracellular resource availability and phage production. Burst size, lysis time, and productivity are plotted against the intracellular RNA contents (mass %) at the time of infection in (a), (b) and (c), respectively. (d) Productivity is plotted against intracellular DNA contents (mass %). $R^2$ values were obtained from linear regression and the $P$-values determined from $F$-tests on the significance of the slope.
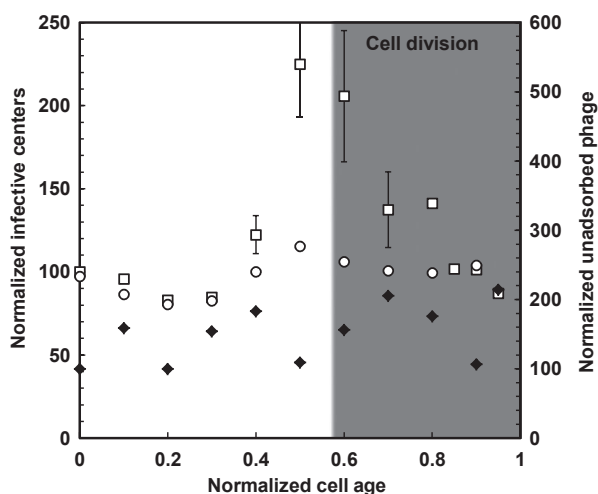


**Fig. 3.** The normalized total infective centers (□) and the number of unadsorbed phage (◆) measured after infecting synchronized cultures with bacteriophages. For reference, infective center data collected from an unsynchronized batch experiment operated under the same conditions are also shown (○). To compare data collected from a large number of experiments with slightly different initial conditions, the values from each experiment have been set to 100 at a cell age of zero. Error bars represent the standard deviation of replicates from at least three separate experiments.

under similar conditions. Note that although the data was measured in PFU mL$^{-1}$, the values shown in Fig. 3 have been normalized to compare data collected from a large number of experiments with slightly different initial conditions. This was done by setting the value of infective centers (or unadsorbed phage) to 100 at a cell age of zero. Although the cell cycle has no significant effect on

phage adsorption (the number of unadsorbed phage remaining constant throughout the cell cycle), it has a dramatic impact on the creation of infective centers and the outcome of phage infection. The number of infective centers measured in samples infected immediately preceding cell division is twice the values measured in samples from young cells infected immediately following cell division. The unsynchronized cell data collected shows only mild fluctuations throughout the cycle, although these do have a small maximum at the same point as the synchronized curve.

## Discussion

The results presented here demonstrate that the cell cycle is a major contributing factor to the outcome of a bacteriophage infection. Infections of larger cells late in the cell cycle leads to significant increases in productivity during infection (Fig. 1c) as well as increasing the number of infections resulting in adult phage release (Fig. 3). Although it is clear that the cell cycle strongly influences phage infection, the mechanism of this phenomenon is less apparent. In this study, all experiments were controlled for medium composition, temperature, specific growth rate and other major fermentation variables. Consequently, the trends observed can be considered to be a direct result of the stage in the cell cycle.

Phages require numerous nucleic acid precursors, protein precursors, and translation machinery for efficient virus production (You *et al.*, 2002). Therefore the intracellular concentration of these crucial resources must influence the productivity of a phage infection. An increase in phage productivity necessitates higher rates of

protein production and phage assembly within the cell. Consequently, a large cell could not lead to increased phage productivity by virtue of size alone. Indeed, You et al. (2002) suggested that a dilution of the interacting proteins during phage assembly in extremely large cell volumes can decrease phage productivity during infection. Intracellular resources such as RNA, ribosomes, and other forms of cell metabolic machinery must be available in larger quantities and specifically for phage reproduction in order for the productivity to increase. In fact, intracellular RNA concentration was observed to correlate strongly and significantly with an increase in burst size, decrease in lysis time, and increase in phage productivity during infections of synchronized cultures (Fig. 2a–c). On the other hand, the concentration of DNA in the cell at the time of infection was less important and did not correlate significantly with phage productivity (Fig. 2d).

The mass fraction of RNA in an individual cell varied with the cell cycle (Figs S2 and S3), reaching a maximum in samples taken immediately prior to cell division. Replication within the host cell involves hijacking the host cell RNA polymerases and replication machinery and reprogramming them for virion production. For example, in the case of phage T4, the host cell polymerase is relied upon throughout the entire infection to initiate transcription of early, middle and late phage proteins (Mosig & Hall, 1994; Roucourt & Lavigne, 2009). Host cell proteins are also recruited for replication of the phage genome (Roucourt & Lavigne, 2009). Higher levels of these host cell ribosomes, polymerases, and other metabolism-related molecules thus increase the rate of phage production (Fig. 2c).

The influence of the cell cycle on the likelihood of a productive infection was an unexpected result (Fig. 3). From the experimental data, it can be concluded that the adsorption dynamics were not significantly affected by the cell cycle, as the number of free phages was the same after 5 min of incubation with cells at any stage in the cell cycle. However, it is not obvious why the number of infections leading to phage replication would be lower for recently divided cells than for late-cycle cells. The reduced availability of cellular resources could be a factor, although one would think that this would merely limit the productivity of the infection, not reduce the productivity to zero.

The unadsorbed phage data shown in Fig. 3 were determined after lysing cells with chloroform, which results in the release of any reversibly attached phage (Zonenstein et al., 2010), indicating that irreversible phage attachment was not affected by the cell cycle. Since DNA ejection and penetration are initiated by conformational changes following irreversible attachment, it is unlikely that this step was affected. There are a variety of abortive infection (Abi) systems that bacteria employ which simultaneously inhibit mature phage production after DNA injection and kill the cell (Chopin et al., 2005). These systems are present in order to limit the propagation of a phage infection. However, most Abi systems of this type are found in Lactococcus species and encoded by a plasmid (Chopin et al., 2005). Similar systems exist in E. coli, such as the Lit and Prr phage exclusion systems, but they are generally encoded by a prophage or only exist in specific strains (Snyder, 1995). Also, these systems would need to operate preferentially in recently divided cells. It is thus unlikely that such a system is behind the observations noted in this study. One possibility is that the expression level of early transcripts for lytic development is affected by the cell cycle. A study on phage λ found evidence that under-expression of certain early proteins (O and P) could reduce lytic development in minimal media as a response to physiological conditions of the host (Gabig et al., 1998). It is possible that a similar phenomenon is taking place here in response to physiological conditions of the host at different points in the cell cycle.

Interestingly, a comparison of synchronized cells with unsynchronized cells (grown in batch) shows that the intra-cycle variability of infective centers increased significantly with the synchronization process (Fig. 3). When performing the experiment on an unsynchronized culture grown under comparable conditions, the number of infective centers showed only modest changes. These results strongly indicate that cell cycle can influence the fate of a virulent phage after infection of the host cell. Further study comparing the cellular metabolic machinery before and after binary fission is needed to understand the underlying mechanisms behind this observation. In addition, a similar study with phage λ may shed new light on the effects of the cell cycle on the lytic/lysogeny decision in temperate phages. From the results presented here, it would seem there exists a selective pressure to lyse the largest cells in the culture, presumably due to the availability of intracellular resources.

## Conclusion

The present study provides for the first time a detailed analysis of the effect of the cell cycle on the outcome of phage T4 infection, in terms of both the rate of phage replication and the successful production of progeny itself. The burst size and lysis time were found to fluctuate significantly during the cell cycle with maxima and minima, respectively, for cells infected before the onset of cell division. Examination of host intracellular RNA at the time of infection shows that it is a significant parameter correlating with the productivity of phage infections. Finally, infections at different stages in the cell cycle

significantly affected the proportion of abortive infections. This work demonstrates the important influence the host cell cycle has on a virulent bacteriophage.

## Acknowledgements

## References

Adams MH (1959) *Bacteriophages*. Interscience Publishers, New York, NY.

Blumenthal LK & Zahler SA (1962) Index for measurement of synchronization of cell populations. *Science* **135**: 724.

Brown WA (2001) The self-cycling fermentor – development, applications, and future opportunities. *Rec Res Dev Biotechnol Bioeng* **4**: 61–90.

Brown WA & Cooper DG (1991) Self-cycling fermentation applied to *Acinetobacter-calcoaceticus* Rag-1. *Appl Environ Microbiol* **57**: 2901–2906.

Chopin MC, Chopin A & Bidnenko E (2005) Phage abortive infection in lactococci: variations on a theme. *Curr Opin Microbiol* **8**: 473–479.

Dawson PSS (1972) Continuously synchronized growth. *J Appl Chem Biotechnol* **22**: 79–103.

Ellis EL & Delbruck M (1939) The growth of bacteriophage. *J Gen Physiol* **22**: 365–384.

Gabig M, Obuchowski M, Wegrzyn A, Szalewska-Palasz A, Thomas MS & Wegrzyn G (1998) Excess production of phage lambda delayed early proteins under conditions supporting high *Escherichia coli* growth rates. *Microbiology* **144**(Pt 8): 2217–2224.

Hadas H, Einav M, Fishov I & Zaritsky A (1997) Bacteriophage T4 development depends on the physiology of its host *Escherichia coli*. *Microbiology* **143**: 179–185.

Helmstetter CE & Cummings DJ (1963) Bacterial synchronization by selection of cells at division. *P Natl Acad Sci USA* **50**: 767–774.

Kutter E, Kellenberger E, Carlson K *et al.* (1994) Effects of bacterial growth conditions and physiology on T4 infection. *Molecular Biology of Phage T4*, (Karam JD, Ed), pp. 406–418. American Society for Microbiology, Washington, DC.

Mosig G & Hall DH (1994) Gene expression: a paradigm of integrated circuits. *Molecular Biology of Bacteriophage T4* (Karam JD, ed), pp 127–131. American Society for Microbiology, Washington, DC.

Rabinovitch A, Hadas H, Einav M, Melamed Z & Zaritsky A (1999) Model for bacteriophage T4 development in *Escherichia coli*. *J Bacteriol* **181**: 1677–1683.

Roucourt B & Lavigne R (2009) The role of interactions between phage and bacterial proteins within the infected cell: a diverse and puzzling interactome. *Environ Microbiol* **11**: 2789–2805.

Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sauvageau D & Cooper DG (2010) Two-stage, self-cycling process for the production of bacteriophages. *Microb Cell Fact* **9**: 81.

Sauvageau D, Storms Z & Cooper DG (2010) Synchronized populations of *Escherichia coli* using simplified self-cycling fermentation. *J Biotechnol* **149**: 67–73.

Sheppard JD & Cooper DG (1990) Development of computerized feedback-control for the continuous phasing of *Bacillus subtilis*. *Biotechnol Bioeng* **36**: 539–545.

Snyder L (1995) Phage-exclusion enzymes: a bonanza of biochemical and cell biology reagents? *Mol Microbiol* **15**: 415–420.

St-Pierre F & Endy D (2008) Determination of cell fate selection during phage lambda infection. *P Natl Acad Sci USA* **105**: 20705–20710.

Wang IN (2006) Lysis timing and bacteriophage fitness. *Genetics* **172**: 17–26.

You L, Suthers PF & Yin J (2002) Effects of *Escherichia coli* physiology on growth of phage T7 *in vivo* and *in silico*. *J Bacteriol* **184**: 1888–1894.

Zonenstein Y, Zaritsky A, Merchuk J, Einav M & Enden G (2010) The initial adsorption of T4 bacteriophages to *Escherichia coli* cells at equivalent concentrations: experiments and mathematical modeling. *Biochem Eng J* **48**: 225–229.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Cycle lengths for the six different SCF runs used to generate synchronized cultures for this study.

**Fig. S2.** Intracellular RNA levels were calculated by comparing the total RNA concentration of cultures growing in a SCF cycle to the cell dry weight.

**Fig. S3.** Intracellular RNA (mass percent) of the cells as a function of cell age.