Supplementary data for:

Real-time kinetics of restriction-modification gene expression after entry into a new host cell

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In this supplement, we present evidence that M13 infections can lead to oscillatory behavior under certain circumstances, which would interfere with the use of this system for gene expression kinetic studies.

SUPPLEMENTAL METHODS
Unless otherwise specified here, all methods were the same as those described in the text of the paper.

M13 infection assay
M13mp19 phage infection of \textit{E. coli} TOP10F\textsuperscript{'} was monitored by counting the blue/white colony ratios, from samples plated onto LB agar supplemented with X-gal and IPTG at 40 \(\mu\text{g/ml}\) and 300 \(\mu\text{g/ml}\), respectively. Prior plating the cells were rapidly chilled on ice and subsequently 3 times washed with 2 volumes of PBS buffer to remove the free phage. Phage titers were determined by plaque assay on the same strain, using the top agar overlay technique \textsuperscript{(1)}. Due to potential instability of M13 clones \textsuperscript{(2)}, the phage stocks were prepared from non-passaged, originally-isolated M13 RF DNA via transfection.

M13 replicative form (RF) accumulation assay
Total DNA was purified from each sample \textsuperscript{(3)}. Prior to real-time PCR, all DNA preparations were adjusted to the same DNA concentration (1.5 \(\mu\text{g/\mu l}\)) and then diluted 1:200. The real-time PCR as well as the data analysis were performed as in the main text.

SUPPLEMENTAL RESULTS AND DISCUSSION
M13 bacteriophage infection model oscillates in the absence of tetracycline
As described in the associated paper, we chose to use a non-lytic phage model to study \textit{PvuII} gene establishment in a new host. In our initial pilot M13 infection studies, samples were taken every 5 min, through 70 min post-infection. Actively-growing \textit{E. coli} \textit{F}\textsuperscript{'} cells were infected, exactly as in experiments described in the associated paper (Figure S1), with M13mp19 or M13cat at a multiplicity of infection (MOI) of 5, but in LB lacking tetracycline. The \textit{F}\textsuperscript{'} plasmid in \textit{E. coli} TOP10F\textsuperscript{'} contains the \textit{tet} gene, but we had originally omitted this antibiotic as growth (even of resistant cells) is
slowed by tetracycline, and antibiotics can also interfere with β-galactosidase assays (4). Furthermore, infection with M13 makes E. coli cells more fragile (5-7), and the combination of filamentous phage and low doses of certain antibiotics, including tetracycline (30 µg/ml), can inhibit growth or kill otherwise-resistant hosts (8). As shown here, this system showed cycling behavior. Tetracycline greatly reduced this cycling, and was used in experiments shown in the accompanying paper.

We measured the number of cells in the culture by plating to determine colony forming units (CFU), and the phage number by determining plaque forming units (PFU) on a bacterial lawn. As M13mp19 carries lacZα, while the host strain carries lacZΔM15 (9), X-gal and IPTG were included in the CFU plating media to distinguish infected (smaller, blue colonies) from uninfected cells (larger, white colonies).

Our initial experiments used an MOI of 5 PFU per CFU at a cell density of 7×10⁷ CFU/ml. This corresponds to an “actual MOI” (10) of 0.37 if 30 min are allowed for the infection period. The actual MOI reflects the binding constant for phage and cell target, the cell concentration, and the time allowed. However, three-quarters of the cells were infected within 5 min (Figure S2A). Poisson analysis suggests that 75% infection is consistent with an effective MOI just under 2.

Unexpectedly, the number of M13-infected cells appeared to cycle with a period of about 20 min (Figure S2A). The uninfected (Lac-) cells did not show significant cycling. By 60 min post-infection, most of the infected cells were dead, or at least incapable of forming colonies. This could be a source of noise in our gene expression measurements: if these cells have lysed, they would not contribute to our measurements; but if they are in a viable but non-culturable state (11-13), they would contribute to these measurements. A large fraction of the infected cells showed an unstable colony phenotype. The blue colonies often had white enlarged sectors, suggesting phage loss, with or without white papillation patterns (Figure S2F). Usually the colonies presented a combination of these patterns.

M13 PFU/ml from the same culture showed comparable time phases of phage production (Figure S2B). The first substantial release of phage began about 20 min post-infection, in the agreement with measurements of the M13 infection-to-release time of 15-20 min (14,15). After this, PFU/ml nearly doubled (15-30 min post-infection). After this, phage production leveled off and phage levels did not change significantly (Figure S2B).

M13 phage transcript levels were determined for the growing culture infected with M13mp19 phage (Figure S2C). Total RNA from the culture samples was isolated and prepared for quantitative analysis via QRT-PCR (Materials and Methods). We probed for M13 mRNA from genes II and VIII. M13 gII is one of the earliest transcripts, the product of which is involved in DNA replication (16), while gVIII is the most abundant mRNA and specifies the major capsid protein (17). We measured the relative mRNA fold change with respect to the very initial level (one min after infection) and normalized to E. coli recA. Previous microarray analysis indicates that M13 infection does not significantly alter recA expression (18). Phage transcripts (gII and gVIII) rose detectably about 20 min after infection and the levels of phage transcripts kept the same 20 min periodicity over the monitored time course of 70 min (Figure S2C) consistent with monitoring the infection ratio (Figure S2A).
The same periodicity phenomenon was observed for cells infected with M13cat phage (Figure S2D). CAT protein was detectible by ELISA about 20 min post-infection and showed the same cycling phases.

The accumulation of the replicative form (RF) DNA was also measured over the course of the infection with M13pvullwt (Figure S2E). The same cycling was seen, reaching the first peak about 40 min post-infection. Aside from the cycling, overall levels of RF DNA match previous observations, where 8 min after infection, the RF level was ~1 copy per cell, but by 40 min this reached ~35 copies per cell (14).

As shown in the accompanying paper, when tetracycline (for which the F’ carries resistance) is present, the cycling is not seen. The tetracycline presumably eliminates or severely reduces the fraction of cells that have lost the F’, and thus fail to produce the pili that serve as the receptor for filamentous phages such as M13 (19). Phage-resistant mutants can also arise with modified or inactivated pili (20,21). Complex population dynamics may result even among infected cells, as suggested by Figure S2F. M13-infected cells can resist superinfection by inducing pilus retraction (22), and elimination of the F’ factor can be promoted by M13 (23).

Effects of “short circuiting” on plaque morphology.
In the accompanying paper, one way that we tested the physiological relevance of the M13-based model for introducing PvuII genes into a population of cells was to show that a “short circuiting” phenotype is maintained. Host cells with pre-expressed (wt) C.PvuII cannot be transformed with the intact PvuII R-M system; (24,25) this is interpreted as resulting from expression of REase prior to protective methylation of the new host’s chromosome. The EOP of M13 carrying the intact PvuII R-M system is reduced by about three-fold relative to cells having no pre-expressed C.PvuII (Fig. 3 in accompanying paper). The reductions in EOP were correlated with much smaller plaque size than were seen in control infections (Figure S3). These observations suggest that more-severe growth restriction (and possibly cell death) occurs after infection of cells with pre-expressed pvullC, limiting the production of progeny phage.

Supplemental references

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**Figure S1.** Infection of *E. coli* by M13 phage carrying the PvuII R-M system. *E. coli* TOP10F’ was the host strain for infections with recombinant M13 phages (Table 1). Cells were grown overnight in LB medium, supplemented with tetracycline to maintain the F’ episome. After dilution the culture was grown to OD_{600nm} = 0.23, then split into four equal portions. Of these, one was infected with M13pvullwt (C.PvuII+ phenotype, black diamonds), one with M13pvullEsp19 (C.PvuII– phenotype, grey diamonds), one with M13mp19 (vector control; white diamonds), and the fourth was an uninfected control (circles). The multiplicity of infection (MOI) was 5 PFU/CFU. Triplicate samples were collected over a period of 70 min. The first replicate sample in each case was used for total RNA isolation, the second was used for genomic DNA isolation and the third was sonicated for endonuclease activity assays.
Figure S2. M13mp19 infection of *E. coli* TOP10F'. (A) Cultures were infected at an MOI of 5 PFU/ CFU. Due to a-complementation, infected cells are Lac+ and turn blue on agar containing X-gal. Filled circles indicate CFUs of blue, infected cells; open circles indicate CFUs of white uninfected cells. (B) Phage production expressed as the plaque-forming units (PFUs). (C) The relative levels of M13 phage transcripts for gII (filled circles) and gVIII (open circles). The RNA isolation and QRT-PCR were performed as described in Materials and Methods. (D) Chloramphenicol acetyltransferase (CAT) production after infection with M13cat phage (filled diamonds), at MOI=5, vs. uninfected cells (open diamonds). CAT levels, determined via immunoassay as described in Materials and Methods, are expressed as ng of protein per 1 ml of culture at OD_{600nm}. (E) The relative levels of M13 phage DNA (RF, replicative forms) determined for gII. The RF DNA isolation and Q-PCR were performed as described in Materials and Methods. (F) Examples of *E. coli* Top10F' M13mp19-infected colonies grown on LB agar plates supplemented with IPTG, X-gal for 2 days. Note the numerous white papillae and/or sectors on the otherwise-blue colonies.
Figure S3. Effect of active C.Pvull of plague size formation. For each combination of pre-expressed *pvull*Cwt or *pvull*CEsp19 (Figure 3A), the M13 phage EOP reduction correlated with the much smaller plaque size (A) in comparison to the typical M13 plague (B), both grown 2 days in 37°C. (A) host cells pDK200 (pre-expressed wt *pvull*C) infected with M13pvullwt phage; (B) the same host cells but infected with M13mp19 phage (no *pvull*C)