Microbiology (2013), **159**, 507–514 **DOI** 10.1099/mic.0.064790-0

A_2 expression and assembly regulates lysis in Qβ infections

Catrina A. Reed, Carrie Langlais,† Ing-Nang Wang‡ and Ry Young

Department of Biochemistry and Biophysics, Texas A&M University, 2128 TAMU, College Station, TX 77843-2128, USA

Correspondence
Ry Young
ryland@tamu.edu

Received 7 November 2012
Revised 29 December 2012
Accepted 5 January 2013

The capsids of ssRNA phages comprise a single copy of an ~45 kDa maturation protein that serves to recognize the conjugative pilus as receptor, to protect the ends of the viral RNA and also to escort the genomic RNA into the host cytoplasm. In the *Alloleviviridae*, represented by the canonical phage Qβ, the maturation protein A_2 also causes lysis. This is achieved by inhibiting the activity of MurA, which catalyses the first committed step of murein biosynthesis. Previously, it was shown that Qβ virions, with a single copy of A_2, inhibit MurA activity. This led to a model for lysis timing in which, during phage infection, A_2 is not active as a MurA inhibitor until assembled into virion particles, thus preventing premature lysis before a sufficient yield of viable progeny has accumulated. Here we report that MurA inactivates purified Qβ particles, casting doubt on the notion that A_2 must assemble into particles prior to MurA inhibition. Furthermore, quantification of A_2 protein induced from a plasmid indicated that lysis is entrained when the amount of the lysis protein is approximately equimolar to that of cellular MurA. Qβ *por* mutants, isolated as suppressors that overcome a *murA*^{rat} mutation that reduces the affinity of MurA for A_2, were shown to be missense mutations in *A*$_2$ that increase the translation of the maturation protein. Because of the increased production of A_2, the *por* mutants have an attenuated infection cycle and reduced burst size, indicating that a delicate balance between assembled and unassembled A_2 levels regulates lysis timing.

INTRODUCTION

Three lytic phages with single-strand nucleic acid genomes – *Alloleviviridae* (ssRNA; Qβ), *Leviviridae* (ssRNA; MS2) and *Microviridae* (ssDNA; ϕX174) – are known to effect lysis by expression of a single gene without muralytic activity (Bernhardt *et al.*, 2002). Of these, two cause lysis by inhibiting an essential enzyme in the murein precursor pathway: Qβ A_2 inhibits MurA (Bernhardt *et al.*, 2001) and ϕX174 E inhibits MraY (Bernhardt *et al.*, 2000). In both cases, lysis occurs when the host cell attempts to septate in the absence of murein synthesis. The third, MS2 L, effects lysis by an unknown mechanism.

Qβ has long been one of the paradigm experimental systems for studying viral RNA-dependent RNA polymerase and RNA evolution, in part because of its simplicity (Blumenthal & Carmichael, 1979; Domingo *et al.*, 1978; Domingo & Holland, 1997; Drake, 1993). The Qβ genome consists of only three genes, encoding four proteins: maturation or A_2, coat, read-through coat or A_1 and replicase (Fig. 1a). A single copy of A_2 is mounted on the Qβ virion, comprising a T=3 capsid containing ~165 copies of coat, ~15 copies of A_1 and the 4.2 kb ssRNA (van Duin & Tsareva, 2006). Among the three simple phage systems, the Qβ lysis system is unique in that the lysis protein, A_2, has other functions: it protects the genomic RNA from RNase degradation (Weber *et al.*, 1975), mediates specific adsorption to the F-pilus and chaperones the genome into the host cytoplasm (Kozak & Nathans, 1971). As the other functions of A_2 require binding to Qβ RNA, the phage particle and the host F-pilus, an assortment of potential regulatory modes are possible in the infected cell. A_2 expression is tightly regulated by extensive RNA secondary structure surrounding its translational initiation region (Beekwilder *et al.*, 1996; Groeneveld *et al.*, 1995) (Fig. 1b), and *in vitro* translation experiments indicate that A_2 is produced only from nascent transcripts (Robertson & Lodish, 1970; Staples *et al.*, 1971).

Winter & Gold (1983) revealed another function for this protein by showing that induction of *A*$_2$ cloned on a medium copy plasmid was necessary and sufficient to induce host lysis. Nearly 20 years later, Bernhardt *et al.* (2001) discovered that induction of a plasmid-borne cDNA

†Present address: Rho, Inc., 6330 Quadrangle Drive, Chapel Hill, NC 27517, USA.

‡Present address: Department of Biological Sciences, University at Albany, 1400 Washington Avenue, Albany, NY 12222, USA.

Four supplementary figures, three supplementary tables and supplementary methods are available with the online version of this paper.

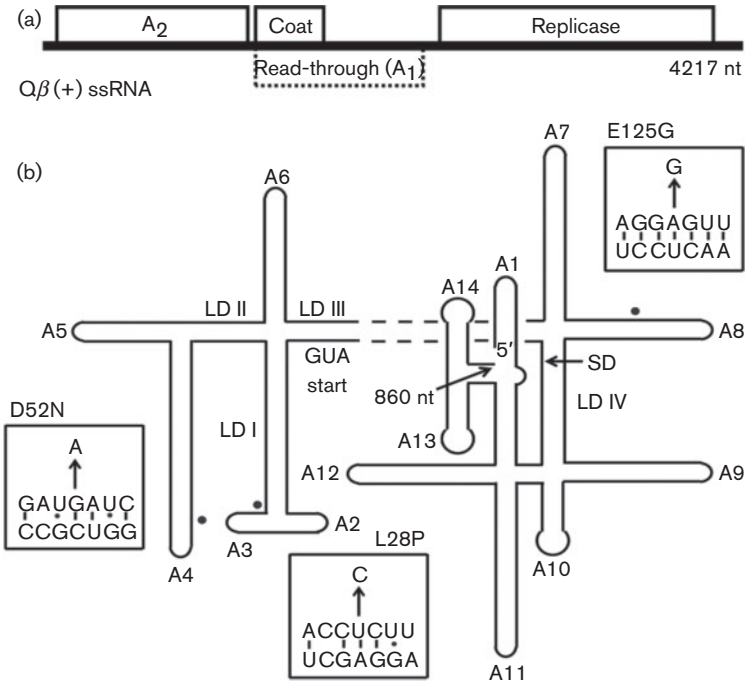


Fig. 1. Qβ genome. (a) Qβ is a (+) ssRNA phage with a 4.2 kb genome. Replication of the chromosome is dependent on encoded replicase and four additional host proteins. The Qβ capsid is composed of three gene products: coat, the major virion structural protein; A_1, a minor component translated from read-through of the leaky coat UGA stop codon; and a single copy of A_2. (b) RNA secondary structure of Qβ 5′-end (nucleotides 1–860). Stem-loops and long-distance interactions (LD) are drawn and labelled according to Beekwilder *et al.* (1996). Single nucleotide substitutions of Qβ *por* mutants are depicted with filled circles, with specific sequence substitutions shown in boxes (arrow). SD, Shine–Dalgarno.

copy of A_2 caused cell-wall synthesis to cease at ~20 min prior to lysis, indicating that A_2 was acting as a 'protein antibiotic' targeting murein biosynthesis. To address the mechanism of Qβ lysis, survivors of A_2 induction were screened for insensitivity to viral infection. Two independent *rat* (*resistance to* A_2) mutants were mapped to *murA*, which encodes UDP-*N*-acetylglucosamine enolpyruvyltransferase, the enzyme catalysing the committed step for the biosynthetic pathway of murein precursors. Both *rat* alleles proved to have a single missense change, Leu138Gln (L138Q). Biochemical studies of A_2 were impeded due to insolubility of the protein apart from the capsid (Bernhardt *et al.*, 2001, 2002). However, MurA activity in a crude lysate was shown to be inhibited by intact Qβ particles. This led to a model for regulation of lysis in Qβ infections in which A_2 does not become inhibitory until fully assembled into the capsid (Hatfull, 2001).

Here we report experiments to test this model, in which we have quantified both MurA and A_2 during the Qβ infection cycle and determined the critical concentration of A_2 required for inhibition of MurA. In addition, the characteristics of Qβ mutants that escape the lysis block imposed by the MurA *rat* mutations are analysed. The results are discussed in terms of a new model for regulation of lysis in Qβ infections.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* strains that were used in this study are listed in Table 1. XL1-Blue was used for all plasmid constructions. XL1-Blue^{rat} cells were used for phage infections. HfrH and HfrH *lacZ*::Tn*5* served as lawns for bacteriophage plating and gradient induction for protein quantification, respectively. ER2738 was used for phage propagation and protein expression. Details of strain and plasmid construction are included in the Supplementary Information, available with the online version of this paper. Primers used for plasmid construction are listed in Table S1. *E. coli* strains were grown under aerobic conditions at 37 °C in standard Luria–Bertani (LB) medium supplemented with 100 μg ampicillin ml^{-1}, 40 μg kanamycin ml^{-1} or 10 μg tetracycline ml^{-1} when appropriate. For Qβ infections, this medium was supplemented with 2 mM $CaCl_2$.

Qβ purification and titre. Qβ particles used in *in vitro* inactivation experiments were purified via caesium chloride gradient centrifugation as described by Strauss & Sinsheimer (1963). Phage for infections was prepared from plate lysates. Approximately 10^5 p.f.u. of Qβ was included in a bacterial soft agar overlay. Plates were incubated at 37 °C for 16 h. The overlay was scraped into a tube with SM medium (Sambrook *et al.*, 1989) and incubated for 1 h. The phage stock was centrifuged at 2800 ***g*** for 20 min and the supernatant was filtered (0.45 μm).

To measure released p.f.u. accumulation, 1 ml infected culture was pelleted, and the supernatant was titrated. Serial dilutions of samples were prepared and 100 μl aliquots were included in a soft agar overlay with a 200 μl aliquot of HfrH grown to mid-exponential phase. After incubation, as above, plaques were counted.

Phage inactivation assay. Approximately 4×10^{11} p.f.u. of Qβ was mixed with 1 μg purified MurAHis protein, as previously described (Reed *et al.*, 2012). A Qβ-only control as well as a sample including 1 μg BSA (Sigma-Aldrich) was prepared in parallel. Samples were brought to 20 μl with buffer (0.1 M Tris, pH 8.0) and incubated at 37 °C for 1 h. Samples were then brought to 1 ml with SM medium. Tenfold serial dilutions were prepared and 100 μl of various dilutions was included in the bacterial overlay for plating comparison. Plates were incubated at 37 °C for 6–8 h and efficiency of plating was assessed. RNase A (Sigma, 1.25 ng μl^{-1}) was also included in the inactivation assay with purified particles and MurA.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype and relevant features	Source or reference
***E. coli* strain**		
XL1-Blue	*recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F′ proAB lacZ*ΔM15::Tn*10*]	Stratagene
XL1-Blue^{rat}	*recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1 murA1 lac* [*F′ proAB lacZ*ΔM15::Tn*10*], *murA1* contains the *murA*^{rat1} mutation corresponding to an amino acid substitution of L138Q	Bernhardt *et al.* (2001)
HfrH	*λ- fhuA*::Tn*10 lacI*^{q} *relA1 spoT1 thi-1*	Laboratory stock
HfrH^{rat}	*λ- fhuA*::Tn*10 lacI*^{q} *relA1 murA1 spoT1 thi-1*	This study
HfrH *lacZ*::Tn*5*	*λ*$^{-}$ *lacI*^{q1} *lacZ*::Tn*5 relA1 spoT1 thi-1*	Laboratory stock
HfrH Δ*murA*::*kan*	*λ- fhuA*::Tn*10 lacI*^{q} *relA1* Δ*murA*::*kan spoT1 thi-1*	This study
pZE12-murA^{Bs}	Δ*murA*::*kan* transduced from strain ZK1745	Brown *et al.* (1995)
ER2738	(F′ *proA*$^{+}$*B*$^{+}$ *lacI*^{q} Δ(*lacZ*)$_{M15}$ *zzf*::Tn*10*) *fhuA2 glnV* Δ(*lac-proAB*) *thi-1* Δ(*hsdS-mcrB*)$_{5}$	New England Biolabs
Plasmid		
pZE12-A$_2$	Q*β* *A*$_2$ cloned under the P$_{LlacO-1}$ promoter, which is IPTG-inducible	This study
pZE12-murA	*E. coli murA* cloned under the P$_{LlacO-1}$ promoter, which is IPTG-inducible	Bernhardt *et al.* (2001)
pZE12-murAHis	An oligo-histidine tag ($G_2H_6G_2$) was adjoined to the C terminus of *murA*	Reed *et al.* (2012)
pZE12-murA^{Bs}	*Bacillus subtilis murAA* cloned under the P$_{LlacO-1}$ promoter, which is IPTG-inducible	This study

Determination of A_2 affinity for MurA. Percentage viability of Q*β* (~0.3 μM) was measured after 30 min incubation with increasing amounts of MurA. Determination of K_i was done by plotting viability versus MurA concentration with the graphing software KaleidaGraph (Synergy Software) and fitting to the following equation (Riley-Lovingshimer & Reinhart, 2001):

$$V=(V_{max}[\text{MurA}]+V_oK_m+V_o[\text{MurA}])/(K_m+[\text{MurA}]) \quad (1)$$

where V_o is the percentage viable when [MurA]=0. V_{max} is m1, V_o is m2 and the K_i (K_m) is m3 (units of μM).

MurA quantification. Quantification of MurA was performed by standard SDS-PAGE and immunoblotting procedures (Gründling *et al.*, 2000). HfrH cellular samples were precipitated by incubating on ice for 30 min with 10 % TCA (trichloroacetic acid); pellets were washed with acetone, resuspended by boiling for 5 min in SDS-PAGE sample loading buffer [50 mM Tris/HCl, 2 % SDS, 5 % glycerol, 100 mM *β*-mercaptoethanol (pH 6.8)] and analysed on an 8 % Tris-Tricine gel. For quantification, a standard of purified MurAHis was prepared. Blots were probed with α-MurA raised against the synthetic peptide, CHGKRPKAVNVRTAP (GenScript), at 1 : 3000 dilution and goat-anti-rabbit secondary antibody (Thermo Fisher Scientific) at a 1 : 3000 dilution. The SuperSignal West Femto developer kit (Thermo Fisher Scientific) was used for chemiluminescence detection. A *murA* knockout in which the chromosomal *murA* was replaced with a kanamycin cassette and complemented with the heterologous *murA* from *Bacillus subtilis* (HfrH *murA*::*kan* pZE12-murA^{Bs}) was used as the negative control for MurA. Strain construction is described in the Supplementary Information.

A_2 induction and quantification. The critical concentration for A_2-mediated lysis was determined by modulating A_2 expression with graded inductions of HfrH *lacZ*::Tn*5* pZE12-A$_2$ at final IPTG concentrations of 0, 12.5, 25, 50, 100 and 1000 μM. This strain is phenotypically LacY^{-} due to polarity of the Tn*5* insertion into *lacZ*; the permease is not expressed, so induction of the *lac* promoter is linearly dependent on inducer concentration (Jensen *et al.*, 1993; Siegele & Hu, 1997). Cell growth was monitored as a function of absorbance (A_{550}) versus time. At various time points, samples were taken for TCA precipitation as described above. SDS-PAGE and immunoblotting was performed, as described above, with a 1 : 10 000 dilution of α-A$_2$ raised against a synthetic peptide, PKLPRGLRFGA (Bethyl Laboratories), and 1 : 3000 dilution of goat-anti-rabbit 2° antibody (Thermo Fisher Scientific). An A_2 standard was obtained by purifying inclusion bodies from cells expressing A_2 protein. The inclusion bodies were processed as described by Palmer & Wingfield (2001) and dissolved in 10 % SDS. The concentration of the purified A_2 protein was determined by amino acid analysis, performed by the Protein Chemistry Laboratory (Texas A&M University).

Quantification of A_2 during an infection was performed as described above, with samples of XL1-Blue cells infected with an input m.o.i. of 1 Q*β* p.f.u. per cell. The amount of A_2 per cell was determined by measuring the total amount of A_2 ml^{-1}, dividing by the total number of cells ml^{-1}, and multiplying by 1.6 to correct for the expected number of uninfected cells based on Poisson statistics.

RESULTS

MurA inactivates Q*β* particles

We were initially interested in attempting to purify a Q*β*-MurA complex. However, when we incubated purified Q*β* (~0.3 μM) with purified MurA (1 μM), we were surprised to find that the virions were quantitatively inactivated (Fig. 2a, b). In contrast, identical incubations with BSA had no effect on plating efficiency (Fig. 2c). Occlusion of virion particles from adsorbing to the F-pilus by MurA was ruled out because the virion-MurA complex is diluted 5×10^5-fold before inclusion in the overlay. We exploited this inactivation reaction to determine that the apparent affinity of virion-associated A_2 for MurA was ~40 nM (Fig. 3). The inactivation reaction was further examined by fractionation on a sucrose step gradient. Analysis of the samples showed

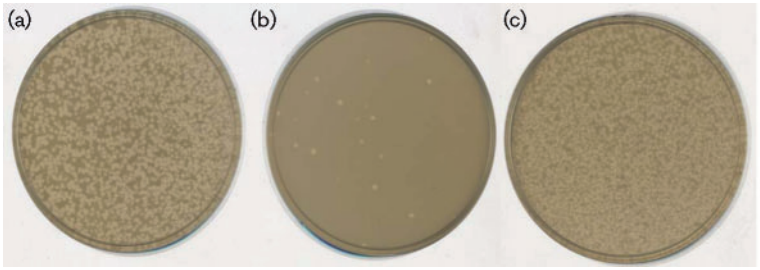
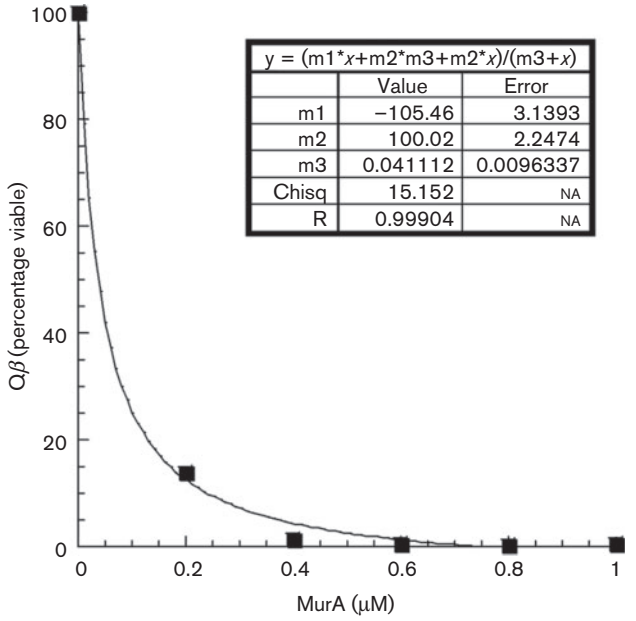


Fig. 2. Qβ particles are inactivated by incubation with MurA *in vitro*. Plating of purified Qβ particles (a) in an HfrH overlay. Incubation of Qβ with MurA (b) or BSA (c) was performed prior to plating.

that a fraction of the genomic RNA became RNase-sensitive in the Qβ sample that was incubated with MurA but not with the virion-only control (Table S2 and Fig. S1). A similar RNase sensitivity has been reported for ssRNA particles that lacked maturation protein (Boedtker & Gesteland, 1975); here, however, the A_2 protein is still present. Thus, the simplest interpretation is that MurA binding causes a conformational change of the maturation protein that inactivates the virion for adsorption to the F-pilus and allows access of RNase to the viral RNA.

Quantification of MurA and A_2 *in vivo*

The finding that MurA can inactivate the virions called into question the previous model that the titration of extant MurA levels by assembled virions accounted for the temporal regulation of lysis in Qβ infections. To begin addressing the question of how A_2-mediated lysis is regulated *in vivo*, it was necessary to quantify both MurA and A_2. The endogenous level of MurA was determined by quantitative Western blotting, using purified MurA as a standard. The result, 390 ± 30 molecules per cell (mean $\pm$ SD of three blots, Fig. S2), is in agreement with another determination of 410 MurA molecules per cell by MS profiling (Ishihama *et al.*, 2008). In view of the affinity measured above (40 nM), at this level of MurA (~400 nM) A_2 should bind stoichiometrically. Accordingly, we estimated the level of A_2 necessary for lysis by performing a gradient induction of an IPTG-inducible plasmid clone. Lysis was observed at 40 min at 100 μM IPTG (Fig. 4). At this level of induction, ~350 and ~500 A_2 molecules accumulate per cell at 15 and 30 min, respectively (Table 2). As it has been reported previously that there is delay of 20 min after the cessation of cell-wall synthesis before lysis is observed (Bernhardt *et al.*, 2001), the lysis time determined here, 40 min, indicates that quantitative inhibition of MurA was achieved at ~20 min, when ~400 A_2 molecules would be expected. Lysis occurs even earlier at higher levels of inducer (Fig. 4).



y = (m1*x+m2*m3+m2*x)/(m3+x)		
	Value	Error
m1	−105.46	3.1393
m2	100.02	2.2474
m3	0.041112	0.0096337
Chisq	15.152	NA
R	0.99904	NA

Fig. 3. Determination of K_i for Qβ inactivation by MurA. Percentage viability of Qβ (~0.3 μM) was measured after 30 min incubation with increasing amounts of MurA. The K_i was determined by plotting viability versus MurA concentration and fitting to equation (1) using the graphical program KaleidaGraph.

Quantification of A_2 accumulation during infection

The context of the viral infection cycle might change the stoichiometry of A_2 and MurA, especially considering that in this framework A_2 has multiple roles. To address this issue, A_2 was quantified during a Qβ infection of the male strain XL1-Blue. The total A_2 concentration was found to exceed that of MurA by 30 min after infection (Table 3 and Fig. 5a), so, as noted above, lysis could be expected at ~50 min. However, culture-wide lysis, monitored both by turbidity and by release of Qβ virions (Fig. 5a, b), was not observed until 80 min, suggesting that cell-wall synthesis was not blocked until ~60 min, when the level of A_2 (1.2×10^3 molecules) is threefold higher than the level of MurA. This suggests that during the Qβ infection cycle, a significant fraction of A_2 is sequestered from binding to MurA.

The *por* mutants produce A_2 more rapidly and cause early lysis

Further evidence addressing the relationship between the rate of A_2 production and lysis timing was obtained from

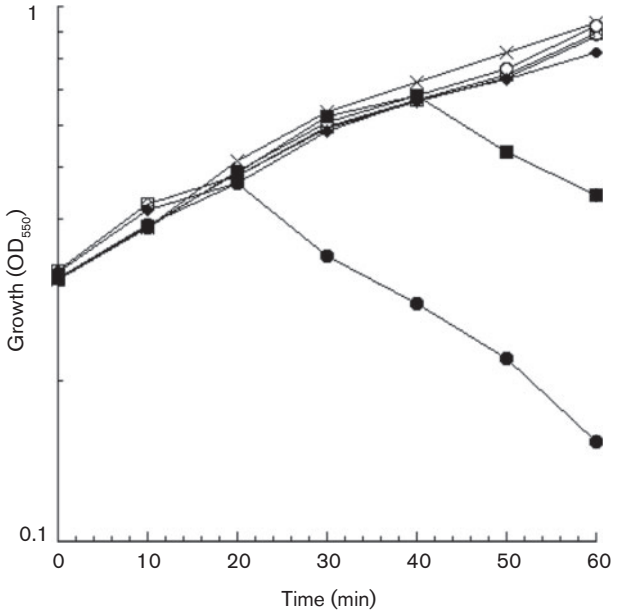


Fig. 4. Plasmid-borne A_2 induces cellular lysis. A_2-mediated lysis was assessed by monitoring growth (OD_{550}) of HfrH *lacZ*::Tn*5* cultures induced with various concentrations of IPTG: pZE12-luc control (1 mM, ×); pZE12-A_2 (uninduced, △; 12.5 μM, □; 25 μM, ○; 50 μM, ◆; 100 μM, ■; 1 mM, ●).

analysis of Qβ *por* (*plates on rat*) mutants (Bernhardt *et al.*, 2001). These mutants were isolated as rare plaque-formers on the $murA^{rat}$ host (Fig. S3). Eight independent mutants were obtained (Table S3); sequence analysis revealed that each had mutations in the 5′ end of the A_2 gene that corresponds to the N terminus of the protein. The simplest interpretation was that missense changes in A_2 could suppress the defect in the inhibition of the $MurA_{L138Q}$ mutant and implied that the N-terminal domain of A_2 carried the lytic determinant. This would be consistent with the observation that the principal differences between

Table 2. Critical concentration of A_2 to induce host lysis

ND, Not determined.

Level of induction (μM IPTG)*	Lysis	A_2 molecules per cell	Time of analysis (min)
0	−	ND	ND
12.5	−	ND	ND
25	−	2.2×10^2	60
50	−	2.3×10^2	60
100	+	3.6×10^2	15
100	+	4.9×10^2	30
1000	+	1.4×10^3	15

*pZE12-A_2.

Table 3. Qβ A_2 quantification

ND, Not determined.

Time (min)	XL1-Blue			
	Qβ A_2WT	Qβ A_2L28P	Qβ A_2D52N	Qβ A_2E125G
15	ND	ND	ND	ND
30	5.1×10^2	1.0×10^3	1.7×10^3	1.3×10^3
45	7.5×10^2	ND	ND	ND
60	1.2×10^3	ND	ND	ND
75	2.9×10^3	ND	ND	ND

the lytic maturation proteins of Qβ and other *Alloleviviridae* and the non-lytic maturation proteins of the *Leviviridae* are in the N-terminal region. However, clones of the A_2^{por} alleles, although fully lytic in the parental host, failed to support inducible lysis in the $murA^{rat}$ background (Fig. S4). Moreover, Western blot analysis of samples taken from infections with the *por* mutants clearly showed that A_2 was accumulating more rapidly than in the parental phage infection (Fig. 5c). These results indicated that the ability of *por* mutants to support plaque formation on the $murA^{rat}$ background is due to a higher rate of synthesis of A_2 *in vivo*, rather than an altered ability to inhibit $MurA_{L138Q}$. Inspection of the secondary structure map of the A_2 mRNA shows that each of the mutations alters a base-pairing element that is predicted to be involved in repressing translational initiation (Fig. 1b). We conclude that the *por* mutations derepress the translational control of A_2.

Based on the previous results with the inducible cDNA clones of A_2 (Fig. 4), we expected that the *por* mutants would show earlier lysis in infections of wild-type cells. We found that this is indeed the case (Fig. 5d), with lysis onset occurring 30–45 min earlier than with wild-type Qβ. With all three *por* alleles, the level of total A_2 production by 30 min was equal to or greater than that achieved by the wild-type phage at 60 min, accounting for the earlier lysis (Table 3 and Fig. 5c). The early lysis also reduces the yield of progeny (Fig. 5b), demonstrating that the normal regulation of the lysis protein is important for the fitness of Qβ.

DISCUSSION

The discovery that two of the paradigm experimental phage systems, the ssDNA phage φX174 and the ssRNA phage Qβ, both achieved host lysis by specifically inhibiting steps in the murein precursor biosynthesis pathway was interesting from the perspective of phage-based design of 'protein antibiotics' (Bernhardt *et al.*, 2001). In addition, these findings also raised the question of how these phages regulated the timing of lysis, and thus the length and fecundity of their infection cycles. The fact that the lytic protein in Qβ, A_2, also served as an essential component of the virion led to the proposal of the simplest possible

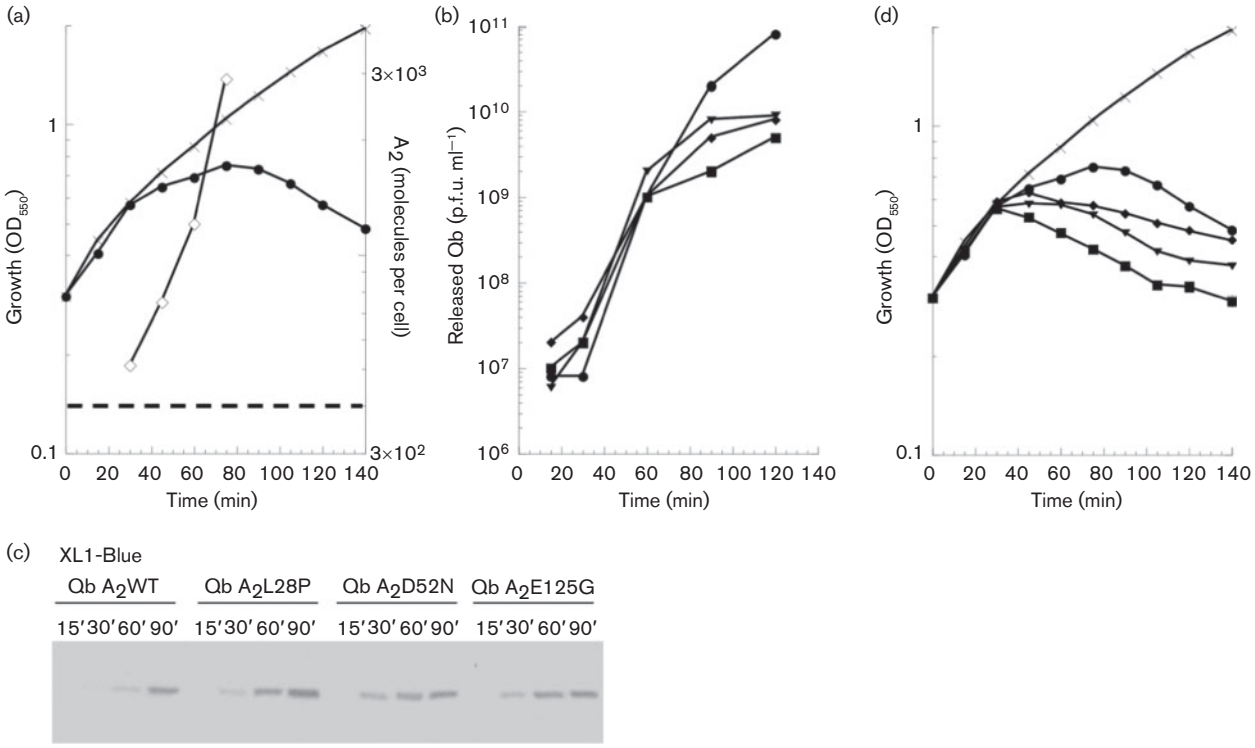


Fig. 5. Qβ and Qβ *por* phenotype of infected cells. (a) Infection of XL1-Blue cells with Qβ. Reduction in cellular mass accumulation is observed starting at 30 min with a maximum at 80 min with phage input m.o.i. of 1. A_2 accumulation (◇) was quantified at intervals during infection. The dashed line represents the number of MurA molecules in XL1-Blue. (b) Qβ has a higher released p.f.u. titre after a single infection cycle than Qβ *por*. (c) A_2 accumulation during infection of XL1-Blue. Loading was normalized to total volume. Samples were taken at the time points listed (min). (d) Qβ *por* mutants have an earlier lysis phenotype in XL1-Blue-infected cells. Qβ infection (a) reproduced with Qβ *por* infections. Qβ-only control (×), Qβ A_2WT (●), Qβ A_2L28P (◆), Qβ A_2D52N (■) and Qβ A_2E125G (▼).

regulatory scheme that would ensure the production of a certain level of virions; that is, the virions themselves, each carrying one copy of A_2, would accumulate until they titrated out the level of cytoplasmic MurA, the target of A_2 (Hatfull, 2001). However, here we have shown that MurA actually inactivates the Qβ virion *in vitro* (Fig. 2), apparently causing conformational changes in A_2 that make the genomic RNA susceptible to exogenous RNase (Fig. S1). Alternative schemes might be based on either a low affinity of virion-associated A_2 for MurA, so that only a small fraction of virions would be inactivated, or that A_2 is produced in a large excess over MurA, so that attrition of infectious virions by contact with MurA would be negligible. Here we have demonstrated that neither of these conditions applies. First, based on the *in vitro* inactivation experiments, the level of MurA in the cell is an order of magnitude higher than needed to saturate A_2 (400 nM MurA compared with 40 nM for the apparent K_d) (Fig. 3). Moreover, graded induction of a cloned *A_2* gene indicates that cell-wall biosynthesis is blocked when the level of A_2 is equimolar with cellular MurA (Fig. 4). Finally, during the infection cycle, A_2 is produced in an amount (1.2×10^3 molecules at the interpolated time of inhibition of cell-wall biosynthesis; Table 3, 60 min) only threefold higher than MurA (~400 molecules).

These considerations suggest that regulation of lysis timing in Qβ infections involves another viral-specific component. Qβ is an extremely simple virus, with only four proteins, and so the candidates for the regulatory component are limited. Here we propose that the regulatory molecule is the Qβ genomic RNA. This notion is based on several lines of evidence. First, *in vitro* gene expression experiments have suggested that initiation of translation of the maturation protein cistron occurs only in nascent RNA molecules that lack inhibitory secondary structures (Robertson & Lodish, 1970; Staples *et al.*, 1971). More recently, it has been proposed that Qβ virion assembly starts with circularization of the viral RNA by a single molecule of A_2 binding both the 5′ and the 3′ ends of the RNA (Dykeman *et al.*, 2011; Shiba & Suzuki, 1981). Based on these observations and the data presented here, we propose a model in which A_2 is synthesized from nascent Qβ RNAs in slight stoichiometric excess. The majority of A_2 molecules are bound to the viral RNA and are thus committed to assembly, whereas a small fraction are free

and able to form the inhibitory complex with MurA. In support of this perspective, we note that the *por* mutants, selected for their ability to overcome the *rat* phenotype of $murA_{L138Q}$, produce A_2 at two- to three-fold higher levels than the parental phage, presumably due to the disruption of local RNA secondary structures. This is necessary, because the L138Q mutation in MurA reduces the affinity for A_2 (Reed *et al.*, 2012). As a consequence, lysis occurs much earlier in the Qβ *por* infections and the average yield of virions is drastically curtailed (Fig. 5b, d). This model predicts that A_2 bound to the viral RNA in the context of the virion assembly pathway is insensitive to MurA. Preliminary experiments looking for protection of the virion by purified Qβ genomic RNA have been unsuccessful (data not shown), suggesting that RNA structures specific to the assembly pathway may be involved. It should be pointed out that this type of system offers the evolutionary capability of changing the lysis timing of the Qβ infection cycle. Presumably, many nucleotide positions in the A_2 cistron can be altered to effect marginal changes in A_2 production, which would then have graded effects on the timing of lysis. Regardless, this model presents a conceptual framework for further analysis of Qβ, which, with its genetic simplicity, is an attractive paradigm system for attempting to achieve quantitative and predictive understanding of a viral infection cycle (Tsukada *et al.*, 2009).

ACKNOWLEDGEMENTS

We thank the Young laboratory members, past and present, for their helpful criticisms and suggestions. We also would like to thank Michelle Lovingshimer for help with K_i calculations. This work was supported by Public Health Service grant GM27099 to R.Y., the Robert A. Welch Foundation and the Program for Membrane Structure and Function, a Program of Excellence grant from the Office of the Vice President for Research at Texas A&M University.

REFERENCES

Beekwilder, J., Nieuwenhuizen, R., Poot, R. & van Duin, J. (1996). Secondary structure model for the first three domains of Qβ RNA. Control of A-protein synthesis. *J Mol Biol* **256**, 8–19.

Bernhardt, T. G., Roof, W. D. & Young, R. (2000). Genetic evidence that the bacteriophage φ X174 lysis protein inhibits cell wall synthesis. *Proc Natl Acad Sci U S A* **97**, 4297–4302.

Bernhardt, T. G., Wang, I. N., Struck, D. K. & Young, R. (2001). A protein antibiotic in the phage Qβ virion: diversity in lysis targets. *Science* **292**, 2326–2329.

Bernhardt, T. G., Wang, I. N., Struck, D. K. & Young, R. (2002). Breaking free: "protein antibiotics" and phage lysis. *Res Microbiol* **153**, 493–501.

Blumenthal, T. & Carmichael, G. G. (1979). RNA replication: function and structure of Qβ-replicase. *Annu Rev Biochem* **48**, 525–548.

Boedtker, H. & Gesteland, R. F. (1975). Physical properties. In *RNA Phages*, pp. 1–28. Edited by N. D. Zinder. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Brown, E. D., Vivas, E. I., Walsh, C. T. & Kolter, R. (1995). MurA (MurZ), the enzyme that catalyzes the first committed step in peptidoglycan biosynthesis, is essential in *Escherichia coli*. *J Bacteriol* **177**, 4194–4197.

Domingo, E. & Holland, J. J. (1997). RNA virus mutations and fitness for survival. *Annu Rev Microbiol* **51**, 151–178.

Domingo, E., Sabo, D., Taniguchi, T. & Weissmann, C. (1978). Nucleotide sequence heterogeneity of an RNA phage population. *Cell* **13**, 735–744.

Drake, J. W. (1993). Rates of spontaneous mutation among RNA viruses. *Proc Natl Acad Sci U S A* **90**, 4171–4175.

Dykeman, E. C., Grayson, N. E., Toropova, K., Ranson, N. A., Stockley, P. G. & Twarock, R. (2011). Simple rules for efficient assembly predict the layout of a packaged viral RNA. *J Mol Biol* **408**, 399–407.

Groeneveld, H., Thimon, K. & van Duin, J. (1995). Translational control of maturation-protein synthesis in phage MS2: a role for the kinetics of RNA folding? *RNA* **1**, 79–88.

Gründling, A., Bläsi, U. & Young, R. (2000). Biochemical and genetic evidence for three transmembrane domains in the class I holin, λ S. *J Biol Chem* **275**, 769–776.

Hatfull, G. F. (2001). Microbiology. The great escape. *Science* **292**, 2263–2264.

Ishihama, Y., Schmidt, T., Rappsilber, J., Mann, M., Hartl, F. U., Kerner, M. J. & Frishman, D. (2008). Protein abundance profiling of the *Escherichia coli* cytosol. *BMC Genomics* **9**, 102.

Jensen, P. R., Westerhoff, H. V. & Michelsen, O. (1993). The use of *lac*-type promoters in control analysis. *Eur J Biochem* **211**, 181–191.

Kozak, M. & Nathans, D. (1971). Fate of maturation protein during infection by coliphage MS2. *Nat New Biol* **234**, 209–211.

Palmer, I. & Wingfield, P. T. (2001). Preparation and extraction of insoluble (inclusion-body) proteins from *Escherichia coli*. *Current Protocols in Protein Science* **70**, 6.3.1–6.3.20.

Reed, C. A., Langlais, C., Kuznetsov, V. & Young, R. (2012). Inhibitory mechanism of the Qβ lysis protein A_2. *Mol Microbiol* **86**, 836–844.

Riley-Lovingshimer, M. R. & Reinhart, G. D. (2001). Equilibrium binding studies of a tryptophan-shifted mutant of phosphofructokinase from *Bacillus stearothermophilus*. *Biochemistry* **40**, 3002–3008.

Robertson, H. D. & Lodish, H. F. (1970). Messenger characteristics of nascent bacteriophage RNA. *Proc Natl Acad Sci U S A* **67**, 710–716.

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

Shiba, T. & Suzuki, Y. (1981). Localization of A protein in the RNA-A protein complex of RNA phage MS2. *Biochim Biophys Acta* **654**, 249–255.

Siegele, D. A. & Hu, J. C. (1997). Gene expression from plasmids containing the araBAD promoter at subsaturating inducer concentrations represents mixed populations. *Proc Natl Acad Sci U S A* **94**, 8168–8172.

Staples, D. H., Hindley, J., Billeter, M. A. & Weissmann, C. (1971). Localization of Q-β maturation cistron ribosome binding site. *Nat New Biol* **234**, 202–204.

Strauss, J. H., Jr & Sinsheimer, R. L. (1963). Purification and properties of bacteriophage MS2 and of its ribonucleic acid. *J Mol Biol* **7**, 43–54.

Tsukada, K., Okazaki, M., Kita, H., Inokuchi, Y., Urabe, I. & Yomo, T. (2009). Quantitative analysis of the bacteriophage Qβ infection cycle. *Biochim Biophys Acta* **1790**, 65–70.

van Duin, J. & Tsareva, N. (2006). Single-stranded RNA phages. In *The Bacteriophages*, pp. 175–196. Edited by R. Calendar. Oxford: Oxford University Press.

Weber, K., Konigsberg, W. & Zinder, N. D. (1975). Proteins of the RNA phages. In *RNA Phages*, pp. 51–84. Edited by N. D. Zinder. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Winter, R. B. & Gold, L. (1983). Overproduction of bacteriophage Q β maturation (A_2) protein leads to cell lysis. *Cell* **33**, 877–885.

Edited by: W. J. J. Meijer