Optimum Threshold Minimizes Noise in Timing of Intracellular Events

HIGHLIGHTS
Mutations in timekeeper protein alter event timing and noise in event timing.

Data show noise in event timings follow a concave up shape with increasing threshold.

Mathematical modeling identifies optimal threshold minimizing noise in event timing.

Results imply that noise in event timing can be a target of natural selection.

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Optimum Threshold Minimizes Noise in Timing of Intracellular Events

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SUMMARY

How the noisy expression of regulatory proteins affects timing of intracellular events is an intriguing fundamental problem that influences diverse cellular processes. Here we use the bacteriophage λ to study event timing in individual cells where cell lysis is the result of expression and accumulation of a single protein (holin) in the Escherichia coli cell membrane up to a critical threshold level. Site-directed mutagenesis of the holin gene generated phage variants that vary in their lysis times from 30 to 190 min. Observation of the lysis times of single cells reveals an intriguing finding—the noise in lysis timing first decreases with increasing lysis time to reach a minimum and then sharply increases at longer lysis times. A mathematical model with stochastic expression of holin together with dilution from cell growth was sufficient to explain the non-monotonic noise profile and identify holin accumulation thresholds that generate precision in lysis timing.

INTRODUCTION

The inherent probabilistic nature of biochemical reactions and low copy numbers of molecules involved result in significant random fluctuations (noise) in protein levels inside isogenic cells inhabiting the same environment (Bar-Even et al., 2006; Cai et al., 2006; Eldar and Elowitz, 2010; Elowitz et al., 2002; Raj and van Oudenaarden, 2008; Taniguchi et al., 2010). Although the origins of stochastic gene expression have been extensively studied across organisms, the impacts of the noisy expression of key regulatory proteins on the timing of intracellular events is underappreciated (Liu et al., 2017; Song et al., 2015; Song and Acar, 2019; Yurkovsky and Nachman, 2013). Identifying regulatory motifs that buffer randomness in the timing of intracellular events has important consequences for disparate cellular processes, such as apoptosis, cell-cycle control, cell differentiation, and sporulation, where precision required for proper system functioning depends on regulatory molecules reaching critical threshold levels at the right time. For instance, cell heterogeneity in concentrations of holin homologues, Bax/Bak (Pang et al., 2011), may determine a cell’s propensity for apoptosis (Santos et al., 2019). Mutations promoting apoptosis resistance may give rise to cells with higher tumorigenic potential (i.e., cancer stem-like cells) (Campbell and Tait, 2018). In another example, proper timing of yeast cell division is ensured by the precise expression of a regulatory protein, Clin, up to a critical threshold level (Schneider et al., 2004). Despite these significant impacts, how cells maintain precision in event timing despite noisy gene expression remains poorly understood.

To address this knowledge gap, we employ the bacteriophage λ as a model system for studying event timing at the single cell level. Here, an easily observable event (cell lysis) is the result of the expression and accumulation of a single regulatory protein (holin) in the Escherichia coli cell inner membrane up to a threshold level (Figure 1A) (Wang et al., 2000; Young, 2014). Once holin surpasses this critical threshold concentration, it nucleates to form large holes in the inner membrane, triggering events that result in the destruction of the cell and the release of phage progeny (Wang et al., 2000; Young, 2014). Since holin nucleation and cell lysis are essentially simultaneous, holin can be said to be the timekeeper of the lysis event (White et al., 2011).

Single-cell observations of holin-induced lysis allows the calculation of both the mean and noise of lysis timing, where noise is quantified using a dimensionless metric, the coefficient of variation (standard deviation divided by the mean). Our prior work revealed incredible precision in lysis timing in the wild-type λ strain: lysis occurs on average at 65 min with a coefficient of variation of less than 5% (Dennehy and Wang, 2011; Singh and Dennehy, 2014).
Despite this precision, different holin mutants exhibited a wide range of means and noise in lysis time (Dennehy and Wang, 2011). The sources of this noise may include variations in the rate of transcription of λ late mRNA, the rate of holin protein translation by host ribosomes, the rate of holin insertion into the plasma membrane, and the holin concentration required for triggering membrane hole formation. In our formulations, the latter two factors were combined into a single parameter, threshold concentration.

To explore how variations in these parameters affect lysis time noise, we mathematically modeled lysis time as the first-passage time for membrane holin levels to cross a critical threshold (Singh and Dennehy, 2014). We employed stochastic gene expression models to derive the exact analytical formulae for the first-passage time moments (Singh and Dennehy, 2014). These formulae were used to generate predictions of how changes in holin transcription and translation efficiencies and holin threshold concentrations can modulate the lysis time mean and variation (Singh and Dennehy, 2014).

The key objective of the work described here is to investigate experimentally how manipulation of the lysis threshold affects the noise in lysis timing. To this end, we systematically altered the amino acid sequence of the holin protein in order to shift (both increase or decrease) the lysis threshold (Ghusinga et al., 2017; Singh and Dennehy, 2014). These amino acid sequence changes may affect holin structure, dimerization and/or oligomerization potential, and/or membrane insertion capacity. Sequence alterations that inhibit holin’s ability to pass into the inner membrane may, for example, increase the threshold, whereas alterations...
increasing holin-holin affinity may decrease the threshold. This contribution studies the effects of these alterations on noise in lysis timing both experimentally and via mechanistic mathematical models to uncover an intriguing insight—precision in timing is enhanced at an intermediate threshold.

RESULTS AND DISCUSSION
Timing of intracellular events is often studied using a first-passage time (FPT) framework that captures the first time a random process crosses a threshold (Co et al., 2017; Ghusinga et al., 2016; Gupta et al., 2018). In our prior work, we formulated lysis timing as an FPT problem (Ghusinga et al., 2017; Singh and Dennehy, 2014). Here, the onset of transcription from λ’s late promoter results in stochastic accumulation of holin within the host cell membrane, and cell lysis is triggered when the total cellular holin concentration reaches a critical threshold (Figure 1D). Our mathematical analysis predicted that noise in timing is inversely proportional to the threshold (Ghusinga et al., 2017; Singh and Dennehy, 2014). The logical progression of this work is to verify this prediction through experimental manipulation of the lysis threshold by altering the holin sequence, which has the effect of altering lysis time mean and noise (Dennehy and Wang, 2011).

It is important to point out that the holin gene S of wild-type λ has two translation initiation sites. Gene expression results in the production of two proteins, holin and antiholin, in a 2:1 ratio ensuring excess holin (Chang et al., 1995). Antiholin has two extra residues, a methionine and a leucine, and acts antagonistically to holin, which has the lysis function (Blasi et al., 1990; Gründling et al., 2000). For the sake of simplicity, we have not considered the effects of antiholin in our model. Although antiholin binding inhibits holin function and thereby delays lysis by several minutes, the biological relevance of this inhibition is still unclear. A simplistic view is that antiholin expression is favored when a delay in lysis is beneficial under adverse growth conditions. However, this has not been conclusively demonstrated so far. Moreover, any mutations in holin would also be incorporated in antiholin as they are encoded from the same gene with different start sites. Thus, to remove any confounding effects of antiholin on lysis timing, we introduced mutations into a strain of λ where antiholin expression has been abolished via the M1L mutation (Table 1).

Site-directed mutagenesis was used to introduce one or two nucleotide substitutions into plasmids bearing the S105 holin allele. The resulting plasmids were used to generate a library of E. coli lysogens differing from the S105 mutant by one or two amino acid substitutions in the holin gene. The optical densities of thermally induced batch cultures of these lysogens were tracked to determine their lysis times (unpublished data). For this study, we selected a subset of twenty holin mutants spanning a wide range of mean lysis times (Table 1). For each mutant strain, we thermally induced and recorded single cell lysis events for ∼100 cells using a microscope-mounted, temperature-controlled perfusion chamber (Figures 1B and 1C, Video S1). This set up resembles a continuous culture system where fresh media is pumped over immobilized cells and waste removed at a constant rate. This allows normal cell growth and metabolism observed as increase in cell length, which facilitates phage multiplication culminating in lysis. The mean lysis times calculated using both the batch culture and single-cell recordings were strongly correlated (Figure S1).

Using a subset of lysogens, we verified that the holin mutations had no effect on holin expression via western blot assays of holin levels in whole-cell extracts (Figure S2). Therefore, any effects on lysis timing can be attributed to shifting of lysis threshold as a result of the amino acid changes in the holin gene. These mutations in holin may affect lysis timing by altering holin-holin affinity, holin accession to the inner membrane, and/or holin nucleation within the inner membrane. To investigate these possibilities further, we compared levels of different holin mutants in the cell membrane. Interestingly, a mutant with short lysis time showed almost 5-fold higher holin levels in the membrane compared with the wild-type (Figure S2). Contrarily, a mutant with long lysis time showed holin levels comparable with the wild-type. In the latter case, the mutated holin might be impaired in the formation of membrane lesions required for lysis, and thus delay lysis. These results suggest that the quality of holin may directly affect the quantity of holin in the membrane and/or its ability to form the membrane lesions critical for lysis. Further biochemical studies may reveal how structural changes in holin affect the different steps leading to cell lysis.

Next, we quantified single-cell FPTs by subtracting 15 min from the recorded lysis times. This 15-min duration accounts for the time delay between lysogen induction and start of transcription from λ’s late promoter (Kobiler et al., 2005; Liu et al., 2013). Recall that simple models predict the noise in FPT to monotonically decrease with increasing lysis threshold (and hence, increasing mean FPT) (Singh and Dennehy, 2014).
Computations of both the mean and noise in FPTs across holin mutants as illustrated in Table 1 reveals an intriguing result—for short-lysis strains decreasing the lysis threshold increases the noise consistent with our previous model. By contrast, the data for long-lysis strains contradicts our simple model; increasing the lysis threshold increases the FPT noise level (Figure 2). The concave-up shape of the plot implies that noise in FPT is minimized at an intermediate threshold. Interestingly, the wild-type genotype resides near the base of this plot suggesting that buffering noise in lysis timing is ecologically relevant and is consistent with the existence of optima in lysis timing (Dennehy and Wang, 2011; Heineman et al., 2007; Wang, 2006; Wang et al., 1996).

To explain this non-monotonic noise profile, we developed an expanded model for noisy holin expression (see section S3). Given that the expressed holin proteins are long-lived and do not degrade over relevant timescales (Gründling et al., 2000; White et al., 2010), their turnover is primarily governed by dilution from cellular growth. As has been shown for E. coli genes (Cai et al., 2006; Chong et al., 2014; Friedman et al., 2006), we consider holin expression occurring in stochastic bursts with holin dilution occurring between two successive burst events. Subsequent analysis of the model predicts the mean FPT as (details in Supplemental information)

| Strain   | Holin Mutations* | n⁵  | Mean FPT (min) (95% Confidence Interval) | FPT CV² (95% Confidence Interval) |
|----------|------------------|-----|----------------------------------------|-----------------------------------|
| JJD3 (WT)| None             | 120 | 48.83 (48.14–49.54)                    | 0.007 (0.0051–0.0089)             |
| JJD5 (S105)| M1L             | 140 | 43.77 (43.02–44.53)                    | 0.011 (0.0089–0.0137)             |
| JJD246   | M1/L/H7D         | 114 | 54.02 (52.86–55.16)                    | 0.013 (0.0097–0.0179)             |
| JJD248   | M1/L/F94C        | 121 | 41.01 (40.23–41.8)                     | 0.012 (0.0094–0.0157)             |
| JJD251   | M1/L/A99V        | 128 | 25.89 (25.24–26.52)                    | 0.02 (0.0158–0.0237)              |
| JJD253   | M1/L/L10M        | 149 | 26.19 (25.51–26.86)                    | 0.026 (0.0183–0.0337)             |
| JJD388   | M1/L25V/N37H     | 99  | 175.09 (163.8–186.1)                   | 0.10 (0.0627–0.1492)              |
| JJD390   | M1/LA11G/Y31H    | 143 | 170.65 (159.9–181.3)                   | 0.13 (0.1116–0.1581)              |
| JJD391   | M1/LA16G/K92Q    | 115 | 125.94 (122–130.1)                     | 0.031 (0.0156–0.0488)             |
| JJD404   | M1/L/21V         | 116 | 15.18 (14.63–15.74)                    | 0.038 (0.0253–0.052)              |
| JJD405   | M1/L/V45G        | 118 | 17.13 (16.7–17.56)                     | 0.02 (0.0147–0.0259)              |
| JJD411   | M1/L/D85G        | 91  | 161.76 (152.4–171.4)                   | 0.086 (0.0699–0.1042)             |
| JJD413   | M1/L/87L         | 158 | 21.32 (20.86–21.78)                    | 0.020 (0.0157–0.025)              |
| JJD414   | M1/L/90l         | 174 | 29.96 (29.2–30.7)                      | 0.029 (0.0229–0.0348)             |
| JJD415   | M1/L/91T         | 166 | 29.11 (28.37–29.85)                    | 0.03 (0.0241–0.0358)              |
| JJD426   | M1/L/G38S        | 100 | 140.26 (134–146.5)                     | 0.053 (0.0374–0.0695)             |
| JJD428   | M1/L/G39S        | 111 | 80.31 (78.23–82.38)                    | 0.02 (0.015–0.0252)               |
| JJD432   | M1/L/S89W        | 132 | 92.74 (90.95–94.61)                    | 0.013 (0.0093–0.0176)             |
| JJD434   | M1/L/D8G         | 104 | 76.09 (74.3–77.93)                     | 0.015 (0.01–0.025)                |
| JJD436   | M1/L/K92N        | 138 | 85.99 (83.86–88.12)                    | 0.021 (0.0116–0.03)               |

Table 1. Mean and Noise in First-Passage Time (FPT) of Isogenic E. coli λ Lysogens
Single-cell FPTs were calculated by subtracting 15 min from the recorded lysis times to account for the time delay between induction and start of transcription from the λ0 late promoter.
WT, wild-type.
* Amino acid substitutions.
⁵ Number of cells observed.
95% CIs after bootstrapping (1,000 replicates).

Computations of both the mean and noise in FPTs across holin mutants as illustrated in Table 1 reveals an intriguing result—for short-lysis strains decreasing the lysis threshold increases the noise consistent with our previous model. By contrast, the data for long-lysis strains contradicts our simple model; increasing the lysis threshold increases the FPT noise level (Figure 2). The concave-up shape of the plot implies that noise in FPT is minimized at an intermediate threshold. Interestingly, the wild-type λ genotype resides near the base of this plot suggesting that buffering noise in lysis timing is ecologically relevant and is consistent with the existence of optima in lysis timing (Dennehy and Wang, 2011; Heineman et al., 2007; Wang, 2006; Wang et al., 1996).

To explain this non-monotonic noise profile, we developed an expanded model for noisy holin expression (see section S3). Given that the expressed holin proteins are long-lived and do not degrade over relevant timescales (Gründling et al., 2000; White et al., 2010), their turnover is primarily governed by dilution from cellular growth. As has been shown for E. coli genes (Cai et al., 2006; Chong et al., 2014; Friedman et al., 2006), we consider holin expression occurring in stochastic bursts with holin dilution occurring between two successive burst events. Subsequent analysis of the model predicts the mean FPT as (details in Supplemental information).
\[
\langle \text{FPT} \rangle = -\frac{1}{\gamma} \log \left( 1 - \frac{X}{X_s} \right), \tag{Equation 1}
\]

where \( \gamma \) is the cellular growth rate, \( X \) is the lysis threshold, and \( X_s \) is the steady-state mean holin concentration reached after a long time if there was no lysis. Moreover, the noise in FPT was derived as

\[
CV_{FPT}^2 = CV_x^2 \left[ \left( e^{\gamma \langle \text{FPT} \rangle} - 1 \right) \left( \gamma \langle \text{FPT} \rangle \right)^2 \right], \tag{Equation 2}
\]

where \( CV_x^2 \) quantifies the extent of stochasticity in holin expression. It is important to point out that \( CV_x^2 \) is determined by the frequency and size of expression bursts (see Equation S13), which in turn depend on transcription and translation rates, respectively. As holin expression levels are unaltered across mutants, we consider \( CV_x^2 \) to be a constant as the mean FPT is varied with increasing lysis threshold. Intriguingly, this formula predicts the timing noise to vary non-monotonically with the mean FPT and provides an excellent fit to the data (black line in Figure 2). A key insight from (2) is that the noise is minimal when the threshold is 55% of the steady-state holin concentration \( X_s \). Intuitively, when the threshold is low, lysis results from a few bursting events, and increasing the threshold suppresses noise through more effective time averaging of burst events. In contrast, at a high threshold close to the steady-state holin level, the holin concentration starts saturating and crossing the threshold becomes a noise-driven event. In this regime, increasing the threshold enhances noise as holin concentration trajectories become even more shallow. In summary, our study uncovers mechanisms for generating precision in the timing of cellular events given the unavoidable constraints of stochastic gene expression and dilution from cellular growth. We first show that genetic variation in event-timing noise exists. We additionally show that this noise follows a consistent pattern where mutations increasing or decreasing the event threshold relative to an optimum value increases noise.

These results suggest that event timing noise may be a feature of cellular event timing systems that is amenable to natural selection. For critical cellular processes, such as apoptosis, cell division, and cell

Figure 2. Noise in Lysis Timing Is Minimized at Intermediate Threshold
Noise in first-passage time (FPT) as quantified using the coefficient of variation squared (CV\(^2\)) is shown plotted against mean FPT across holin mutants. Each point represents an isogenic \( \lambda \) strain with amino acid substitutions (Table 1) affecting the lysis threshold. These mutants show changes in FPT and CV\(^2\) consistent with the model prediction (black line, Equation 2). Equation 2 was fitting to the data considering a 40-min cell doubling time (i.e., E. coli growth at 30°C), with a single-fitting parameter CV\(_x\), which was estimated to be CV\(_x\) = 0.05. Threshold is optimal at the base of the plot where the noise is minimized. WT, \( \lambda \) strain with wild-type \( S \) gene; S105, \( \lambda \) strain bearing the S105 allele (holin); error bars, 95% CIs after bootstrapping (1,000 replicates).
differentiation, the structure and functionality of regulatory molecules may be optimized to buffer randomness in event timing. If true, noise minimization may factor in trade-offs associated with the evolution of regulatory molecules involved in cellular event timing.

Our results imply that the structure and activity of phage λ’s holin molecule itself is not only evolved to trigger host lysis at an appropriate time (Wang, 2006; Wang et al., 1996) but also fine-tuned to ensure precision in lysis timing. In addition to threshold optimization, other aspects of phage λ’s holin lysis system seem designed for noise minimization. The phage λ’s S gene dual start motif, which generates two proteins of opposing function, holin and antiholin, is an archetypal incoherent feedforward circuit. Our previous work showed that, for stable proteins such as holin, feedforward circuits minimize gene expression noise relative to feedback circuits (Ghusinga et al., 2017). In addition, the holin mean burst size (average number of holins produced in a single mRNA lifetime) is estimated to be 1–3 molecules per burst (Chang et al., 1995). Based on our prior FPT moment calculations, such a small protein burst size relative to the event threshold will yield a tight distribution of lysis times (Ghusinga et al., 2017).

Variation in lysis timing, then, may have consequences for phage λ’s fitness. This prediction can be tested in future work by comparing the fitness of mutant strains with same mean lysis timing but different noise levels. Positive results from such competition experiments would provide strong evidence that threshold optimization may be an underappreciated constraint on the adaptive evolution of regulatory timing molecules.

With respect to phage life history, our results present an intriguing mystery. What ecological circumstances induce selective pressure favoring noise minimization in phage λ’s lysis timing? Previous work suggests that, contrary to our findings, phages should experience selection in favor of increased lysis time variance (Baker et al., 2016; Bull et al., 2011). The reason given for this effect is that early bursts contribute more to fitness than late bursts subtract (Baker et al., 2016; Bull et al., 2011). How can we explain the discrepancy? A recent analysis finds that lysis time will converge on an evolutionarily stable strategy (ESS) that minimizes the amount of resources needed by a phage and maximizes phage fitness (Bonachela and Levin, 2014). The impact on viral fitness of any mutations increasing burst size at the expense of increasing the lysis time is positive until host resources begin to limit the burst size (Bonachela and Levin, 2014). Furthermore, the plot of fitness as a function of the latent period has a narrow hump shape and can be maximized around an optimal value (Bonachela and Levin, 2014). In a stable environment, we might expect that selection favors lysis time genotypes that converge on this optimum, thus minimizing noise (Peng et al., 2016).

Two additional points are germane to this problem. First, the model described above is based on the phage and bacteria interacting in a continuous culture. We note that this type of culture likely better reflects the natural habitat of phage λ, i.e., the mammalian gut, than does serial transfer batch culture. Second, the outcomes described are more likely under consistent environmental conditions, such as those experienced during laboratory propagation. This combination of features may further explain why lysis time noise has been minimized in phage λ. It would be interesting to compare our results with that of other phages isolated more recently, especially those with sophisticated lysis systems.

**Limitations of the Study**

In this study, we define threshold concentration as a single parameter (α, see Supplemental Information) that combines both membrane insertion rate as well as holin concentration required for triggering membrane lesion formation. A low threshold could mean high rate of membrane insertion (reaching the threshold concentration faster than the wild-type) and/or highly efficient lesion formation due to a functionally improved holin (i.e., fewer molecules are required for triggering thus lowering threshold concentration). In one instance, we clearly demonstrate a lower threshold due to significantly higher rate of membrane accumulation (UJD405, Figure S2) compared with S105. In this case, although it is safe to assume that high rate of membrane insertion is the primary mechanism, one cannot entirely rule out the possibility that the mutant holin is also more efficient in triggering lesion formation. We do not yet clearly understand if these two processes directly affect each other or they can be mutually exclusive. This will be clear once the amino acids that play crucial roles in the different steps leading to lysis has been accurately identified. Therefore, we are currently unable to predict the effects of individual mutations on model parameters. Along the same lines, it is possible that changes in the holin sequence can render it unstable, which is not considered in the current model.
Resource Availability

Lead Contact
Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, John Dennehy (john.dennehy@qc.cuny.edu).

Materials Availability
All unique/stable reagents generated in this study are available from the Lead Contact without restriction.

Data and Code Availability
Original source data for figures in the paper is available at https://doi.org/10.17632/8t7dxfdgm2.1.

METHODS

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101186.

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AUTHOR CONTRIBUTIONS
Conceptualization, J.J.D. and A.S.; Methodology, S.K., A.S., S.D., and J.J.D.; Investigation, S.K. and T.G.; Formal Analysis, A.S., S.D., and S.K.; Validation, S.K. and T.G.; Visualization, S.K.; Data Curation, S.K.; Resources, I.-N.W. and J.J.D.; Writing – Original Draft, S.K.; Writing – Review & Editing, J.J.D. and A.S.; Project Administration, S.K.; Supervision, J.J.D. and A.S.; Funding Acquisition, A.S. and J.J.D.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

Optimum Threshold Minimizes Noise in Timing of Intracellular Events

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Supplementary Information

Transparent Methods

Bacterial and phage strains

All the bacteria and plasmids used in this study are listed in Table S1. *E. coli* lysogens were cultured in lysogeny broth (LB) at 30°C with rotary shaking (220 rpm).

**Table S1. List of bacterial strains and plasmids used in this study. Lambda lysogens constructed with these strains are listed in Table 1 of the main text.**

| Strain | Genotype | Source |
|--------|----------|--------|
| CGSC#: 6152<sup>a</sup> | *E. coli* MC4100 (λ-) | (Casadaban, 1976) |
| S::Cam | MC4100 (λ cI857 S::Cam) | (Shao and Wang, 2008) |
| JJD3 | MC4100 (λ cI857 S) | (Wang, 2006) |
| JJD5 | MC4100 (λ cI857 S105) | (Wang, 2006) |
| pUCS105R- | pUC18 (λ lysis cassette) | (Shao and Wang, 2008) |

<sup>a</sup>Coli Genetic Stock Center

Construction of plasmids with mutations in holin

Briefly, site-directed mutagenesis was used to generate a panel of mutant λ phages with one or two base pair substitutions in the *S105* allele of the holin gene. Plasmid pUCS105R- carries the λ lysis cassette with the *S105* allele, which has a Leu (CUG) codon in place of the Met1 codon of the *S* gene. This plasmid was used as a template for PCR (Pfu DNA polymerase; Promega, Madison, WI) using megaprimers consisting of 30 to 45-nucleotide homology flanking the altered nucleotides. After *DpnI* treatment to digest the original template, the resulting plasmids were transformed into MC4100 (λ cI857 S::Cam) cells. The cells were spread on LB + Amp (100 μg ml<sup>-1</sup>) plates and incubated at 30°C until colonies were visible. Some of the plasmids thus constructed were further used as templates to generate double mutants.
Transferring mutant holin from the plasmid into the \(\lambda\) phage genome

Transformed MC4100 (\(\lambda\) cI857 S::Cam) cells were grown in 3 ml LB supplemented with ampicillin (100 \(\mu\)g ml\(^{-1}\)) at 30\(^\circ\)C in a rotary shaker. For thermal induction of prophages, the cultures were transferred to a shaker at 42\(^\circ\)C for 15 min and then 37\(^\circ\)C until lysis. The resulting lysate was plated with MC4100 cells to obtain plaque-forming phages resulting from recombination between the prophage and the plasmid. To obtain lysogens, phages obtained from the plaques were used to infect 100 \(\mu\)l of saturated MC4100 culture for 30 min. To this culture, 1 ml LB broth was added and further incubated at 30\(^\circ\)C in a rotary shaker for 1 h. A 100 \(\mu\)l aliquot of this mixture was spread on LB plates supplemented with kanamycin (50 \(\mu\)g ml\(^{-1}\)), and incubated overnight at 30\(^\circ\)C. The lysogens were further screened for ampicillin resistance followed by DNA sequencing to confirm the nucleotide substitutions.

Single-cell lysis time determination

The protocol for determining single-cell lysis times has been described previously (Dennehy and Wang, 2011). Briefly, lysogens were grown overnight in LB at the permissive temperature of 30\(^\circ\)C. Overnight cultures were diluted 100-fold and grown to \(A_{550} = 0.3–0.4\) in a 30 \(^\circ\)C shaking incubator. A 200-\(\mu\)l aliquot of the exponentially growing culture was immobilized to a 22 mm square glass coverslip, which was pretreated with 0.01\% poly-L-lysine (mol. wt. 150 K–300 K; Millipore Sigma, St. Louis, MO) at room temperature for 10 min, and applied to a perfusion chamber (RC-21B, Warner Instruments, New Haven, CT). After assembly, the perfusion chamber was immediately placed on a heated platform (PM2; Warner Instruments, New Haven, CT), which was mounted on an inverted microscope stage (TS100, Nikon, Melville, NY), and infused with heated LB at 30\(^\circ\)C (Inline heater: SH-27B, dual channel heating controller: TC-344B; Warner Instruments, New Haven, CT). The chamber temperature was spiked to 42\(^\circ\)C for 20 min, then maintained at 37\(^\circ\)C until \(\sim95\%\) lysis was observed. Videos were recorded using an eyepiece camera (10X MiniVID™; LW Scientific, Norcross, GA, 10 fps), and the lysis times of individual cells were visually ascertained using VLC™ media player. Lysis time was defined as the time required for a cell to disappear after the temperature was increased to 42 \(^\circ\)C.
S1. Lysis time determination of batch cultures using a plate reader

After sequence confirmation, the lysogens were first heat-induced in batch cultures to assess their lysis times. A 5-µL aliquot of overnight cultures was mixed with 1 mL LB in 24-well plates. Following growth at 30°C for 2 h, the plates were shifted to a 42°C water bath (time 0 for lysis time) for 15 min. After heat induction, the plates were shifted to a pre-warmed plate reader (Synergy™ HT, BioTek® Instruments, Inc., Vermont, USA) at 37°C, which measures A$_{550}$ of the culture every 2 min. This protocol was repeated in triplicate for all 350 lysogens. The complementary cumulative distribution function of the normal distribution was used to fit the A$_{550}$ outputs generated by the plate reader. The estimated mean and standard deviation were defined as the lysis time and spread respectively. FPTs estimated using both the batch culture and single-cell recordings are strongly correlated (Figure S1).

![Figure S1](image)

Figure S1. The FPT measurements using A$_{550}$ and single-cell recordings are strongly correlated. This figure references the strains identified in Table 1 in the main text.

S2. Holin expression

We extracted holin from whole cells as well as cell membranes to compare the relative holin levels in different mutant strains. An exponentially growing culture (A$_{600}$~0.4) at 30°C was induced at
42°C for 20 min. A 5 mL aliquot of the culture was centrifuged to pellet the cells. The pellets were mixed with 2× SDS-PAGE sample buffer, heated at 100°C for 5 min, and loaded on a 4-20% TruPAGE™ precast gel (SIGMA-Aldrich, St. Louis, MO, USA). Another 4 ml aliquot of the culture was sonicated to disrupt the membranes. The membranes were collected by centrifugation at 100,000 × g for 1 h. The pelleted membranes were mixed with 40 μl of ME buffer (10 mM Tris Cl [pH 8.0], 35 mM MgCl₂, 1% Triton X-100) by shaking on a platform shaker for 2 h at 25°C. The extracted samples were centrifuged at 100,000 × g for 30 min to pellet the insoluble fraction. The membrane extracts were mixed with 2× SDS-PAGE sample buffer, heated at 100°C for 3 min, and loaded on to precast gels. After electrophoresis, Western blotting was used to detect holin using a primary antibody (1:1000) raised in rabbits. A secondary antibody (donkey anti-rabbit polyclonal antibody conjugated to horseradish peroxidase [SA1-200, Pierce Chemicals]) was used at a dilution of 1:1000 dilution, and the blot was developed as per the manufacturer’s directions. An average of three preparations was used to estimate holin levels.

**Figure S2.** Total holin levels from whole-cell extracts and membrane fractions. The left and right panels show western blots of membrane fractions and whole-cell extracts, respectively. Bands represent holin extracted from strains S105 (mean LT = 58 min), JJD405 (32 min), and JJD411 (176 min). *, p < 0.05, t-test; error bars, mean ± SEM. Strains shown here reference Table 1 in the main text.
Calculation of noise in the first passage time

We model the expression of holin occurring in intermittent bursts, with burst events arriving as a Poisson process with rate \( k \). Whenever a burst occurs, the total cellular concentration of holin \( x(t) \) at time \( t \) increases by a random amount \( b \):

\[
x(t) \mapsto x(t) + b,
\]

where \( b \) is drawn from an arbitrary positively-valued probability distribution with the first and second moments \( \langle b \rangle \) and \( \langle b^2 \rangle \), respectively. The first moment \( \langle b \rangle \) represents the mean burst size per unit volume. Between two consecutive bursts, the concentration dilutes from cell growth as per the following the deterministic dynamics:

\[
\dot{x}(t) = -\gamma x(t),
\]

where \( \gamma \) is the cellular growth rate. For this hybrid system with stochastic bursts interspersed by first-order decay, the time evolution of the first and second-order moments of \( x(t) \) are given by

\[
\frac{d\langle x \rangle}{dt} = k \langle b \rangle - \gamma \langle x \rangle,
\]

\[
\frac{d\langle x^2 \rangle}{dt} = k \langle b^2 \rangle + 2k \langle b \rangle \langle x \rangle - 2\gamma \langle x^2 \rangle,
\]

(Friedman et al., 2006; Hespanha and Singh, 2005; Singh and Hespanha, 2011). Solving the above differential equations, we get the mean \( \langle x \rangle \) and variance \( \langle x^2 \rangle - \langle x \rangle^2 \) of the holin concentration as a function of time \( t \), assuming there is no holin at the onset of the protein synthesis;

\[
\langle x \rangle = \left[ 1 - e^{-\gamma t} \right] \frac{k \langle b \rangle}{\gamma},
\]

\[
\langle x^2 \rangle - \langle x \rangle^2 = \frac{[1 - e^{-2\gamma t}] k \langle b^2 \rangle}{2 \gamma}.
\]

We formulate the lysis time as the first-passage time

\[
FPT = \min\{t : x(t) \geq X | x(0) = 0\},
\]

or the first time the holin concentration reaches a critical threshold level \( X \), and quantify the noise in the first-passage time using the coefficient of variation squared,

\[
CV^2_{FPT} = (\langle FPT^2 \rangle - \langle FPT \rangle^2)/\langle FPT \rangle^2,
\]

where \( \langle FPT \rangle \) and \( \langle FPT^2 \rangle \) are the first two moments of \( FPT \). \( CV^2_{FPT} \) is related to the fluctuations in the holin concentration as per

\[
CV^2_{FPT} \approx \frac{\langle x^2 \rangle - \langle x \rangle^2}{\langle FPT \rangle^2} \left( \frac{d\langle x \rangle}{dt} \right)^2 \bigg|_{t = \langle FPT \rangle},
\]

(Co et al., 2017). From eq. S5 the mean first-passage time is obtained as
\[ \langle FPT \rangle = -\frac{1}{\gamma} \log(1 - \alpha), \text{ with } \alpha = \frac{X}{x_s}. \]  

S10

Here \( x_s \) denotes the steady-state mean holin concentration and is given by,

\[ x_s = \langle x(t \to \infty) \rangle = \frac{k\langle b \rangle}{\gamma}, \]  

S11

with the underlying assumption in eq. S10 being that the threshold for lysis \( X \) is less than \( x_s \). Using equations (S5), (S6), and (S9), we write down the formula for the noise in \( FPT \).

\[ CV_{FPT}^2 = CV_x^2 \frac{\alpha (2 - \alpha)}{[(1 - \alpha) \ln(1 - \alpha)]^2}, \]  

S12

where, \( CV_x^2 \) is the coefficient of variation squared for the holin concentration at steady state

\[ CV_x^2 = \lim_{t \to \infty} \frac{\langle x^2 \rangle - \langle x \rangle^2}{\langle x \rangle^2} = \frac{\langle b^2 \rangle}{2\langle b \rangle x_s}, \]  

S13

and quantifies the extent of stochasticity in holin expression. The above formula can be rewritten in terms of \( \langle FPT \rangle \) as,

\[ CV_{FPT}^2 = CV_x^2 \frac{e^{2\gamma \langle FPT \rangle} - 1}{(\gamma \langle FPT \rangle)^2}, \]  

S14

and varies nonmonotonically with the mean FPT consistent with experimental data. The optimal value of the mean FPT (in the unit of \( \gamma^{-1} \)), where noise is minimum is \( \gamma(FPT) \approx 0.8 \). The corresponding value of the threshold (in the unit of steady state concentration) is \( \alpha = \frac{X}{x_s} \approx 0.55 \).
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