Threshold regulation and stochasticity from the MecA/ClpCP proteolytic system in *Streptococcus mutans* competence

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Summary

Many bacterial species use the MecA/ClpCP proteolytic system to block entry into genetic competence. In *Streptococcus mutans*, MecA/ClpCP degrades ComX (also called SigX), an alternative sigma factor for the comY operon and other late competence genes. Although the mechanism of MecA/ClpCP has been studied in multiple *Streptococcus* species, its role within noisy competence pathways is poorly understood. *S. mutans* competence can be triggered by two different peptides, CSP and XIP, but it is not known whether MecA/ClpCP acts similarly for both stimuli, how it affects competence heterogeneity, and how its regulation is overcome. We have studied the effect of MecA/ClpCP on the activation of comY in individual *S. mutans* cells. Our data show that MecA/ClpCP is active under both XIP and CSP stimulation, that it provides threshold control of comY, and that it adds noise in comY expression. Our data agree quantitatively with a model in which MecA/ClpCP prevents adventitious entry into competence by sequestering or intercepting low levels of ComX. Competence is permitted when ComX levels exceed a threshold, but cell-to-cell heterogeneity in MecA levels creates variability in that threshold. Therefore, MecA/ClpCP provides a stochastic switch, located downstream of the already noisy comX, that enhances phenotypic diversity.

Introduction

Many species of streptococci can become naturally transformable by entering the transient physiological state known as genetic competence (Johnston *et al.*, 2014; Fontaine *et al.*, 2015). Competence plays a particularly important role for the oral pathogen *Streptococcus mutans*, influencing cell growth, death, interactions with other members of the oral flora and expression of known virulence traits. Bacteriocin production, biofilm formation, acid production and tolerance of acid and oxidative stresses by *S. mutans* all facilitate the competition, persistence and virulence of this organism in the human oral biofilm environment (Lemos and Burne, 2008). All of these traits are linked to the production of ComX (also called SigX), an alternative sigma factor that activates competence genes required for DNA uptake and processing. ComX production is controlled by a pathway that integrates signals received from two quorum sensing peptides (Shanker and Federle, 2016) with environmental cues such as pH (Guo *et al.*, 2014; Son *et al.*, 2015) and oxygen and reactive oxygen species (De Furio *et al.*, 2017), intracellular noise (stochasticity) and positive and negative feedback (Smith and Spatafora, 2012; Reck *et al.*, 2015; Leung *et al.*, 2015; Son *et al.*, 2015; Hagen and Son, 2017). As a result, *S. mutans* competence is a complex and heterogeneous behavior that can be exquisitely sensitive to the extracellular environment and that remains incompletely understood.

Population heterogeneity in *S. mutans* competence is evident from the low efficiency of natural genetic transformation (Li *et al.*, 2001), as well as from observations of cell-to-cell variability in comX gene expression (Lemme *et al.*, 2011; Son *et al.*, 2012; Reck *et al.*, 2015; Hagen and Son, 2017). Transformation efficiency in biofilms is typically less than 0.1% (Li *et al.*, 2001), while even under very favorable conditions no more than 10–50% of cells naturally express comX (Lemme *et al.*, 2011; Son *et al.*, 2012). In addition, the expression of comX can be bimodal or unimodal in the population, depending on the exogenous signals present, the growth phase and the environment (Son *et al.*, 2012; Shields and Burne, 2016). Post-translational regulation of ComX also appears to generate heterogeneity, as high levels of comX mRNA do.
not assure robust activation of comY (Seaton et al., 2011). As with many other bacterial regulatory proteins (Inobe and Matouschek, 2008), ComX levels in S. mutans are modulated post-translationally by an ATP-dependent protease system composed of MecA and ClpCP (Tian et al., 2013; Dong et al., 2014; Dufour et al., 2016). The MecA/ClpCP complex inhibits competence by targeting and degrading ComX, as it does in streptococci of the salivarius, mitis and pyogenic groups (Biornstad and Havarstein, 2011; Boutilry et al., 2012; Wahl et al., 2014; Li and Tian, 2017). However, the function of MecA/ClpCP within the S. mutans competence pathway, and particularly its role in cell-to-cell heterogeneity and the bimodal and unimodal competence behaviors, has not been explored in detail.

Fig. 1 summarizes the competence regulatory pathway in S. mutans (Smith and Spatafora, 2012; Tian et al., 2013; Shanker and Federle, 2016). ComX activates late competence genes that include the nine-gene operon comYA-I, which contains seven genes that are required for transformation (Merritt et al., 2005). Transcription of comX can be triggered by either of two quorum sensing peptides: CSP (competence stimulating peptide) or XIP (SigX-inducing peptide). The efficacy of these peptides is sensitive to environmental factors, including pH, oxidative stress, carbohydrate source and the peptide content of the medium (Son et al., 2012; Son et al., 2015; Moye et al., 2016; De Furio et al., 2017).

CSP is derived from the ComC precursor, processed to a final length of 18 residues and exported to the extracellular medium. S. mutans detects CSP through the ComDE two-component signal transduction system (TCS), which directly activates multiple genes involved in bacteriocin production. ComX, also called SigX, is an alternative sigma factor that directly controls the nine-gene operon comYA-I and other genes required for transformation. The ComRS system includes the peptide ComS and the cytosolic receptor ComR. ComS is the precursor for the quorum-sensing peptide XIP (SigX-inducing peptide). In defined growth medium (lacking assorted small peptides), extracellular XIP is imported by the Ami/Opp permease and interacts with ComR to form a transcriptional activator for both comS and comX. As a result comX is uniformly (population-wide) activated in defined media containing XIP. In complex media, which are rich in assorted small peptides, extracellular XIP does not activate comS and comX; in this case XIP (or possibly its precursor ComS) is proposed to interact with ComR intracellularly, so that both comS and comX are driven by the bistable, intracellular transcriptional feedback loop involving comS and the ComRS complex (Son et al., 2012). As a result comX is heterogeneously (bimodal in population) activated in complex media. The MecA/ClpCP system provides posttranslational regulation of ComX: The adapter protein MecA interacts with ClpC to target ComX for degradation by the protease ClpP. [Colour figure can be viewed at wileyonlinelibrary.com]
biogenesis, secretion and immunity. However, *S. mutans* ComDE does not directly activate *comX*. Instead, the ComRS system is the immediate regulator of *comX* in the mutans, salivarius, bovis and pyogenes groups of streptococci (Fontaine et al., 2015). The ComRS system consists of the cytosolic receptor ComR and the 17 residue peptide ComS, which is processed by an unknown mechanism to form the 7 residue XIP (Mashburn-Warren et al., 2010). Extracellular XIP is imported by the oligopeptide permease Opp and interacts with ComR to form a complex that activates the transcription of *comS* and *comX*. Exogenous XIP induces *comX* efficiently in chemically defined media lacking small peptides (such as FMC or CDM (Mashburn-Warren et al., 2010; Son et al., 2012)), leading to population-wide induction of *comX* at saturating XIP levels. However, XIP elicits no induction of *comX* in complex growth media containing small peptides, possibly owing to peptide competition with XIP for uptake by Opp. Interestingly, the CSP peptide signal has a different action than XIP, as it activates *S. mutans comX* only in complex growth media containing small peptides. It elicits no activity from *comX* in defined media that lacks small peptides, even though CSP stimulates the ComDE TCS (leading to bacteriocin production) under these conditions. In addition, the *comX* response to CSP is bimodal in the population, with no more than 50% of cells expressing *comX* at saturating CSP concentrations (Son et al., 2012).

Consequently, the activation of *comX* in a population of *S. mutans* can exhibit two types of heterogeneity: a unimodal distribution when stimulated by exogenous XIP and a bimodal distribution when stimulated by exogenous CSP. Only the bimodal behavior requires an intact *comS*, whereas only the unimodal behavior requires the oligopeptide permease *opp*. We previously proposed that these different behaviors are two modes of operation of the transcriptional feedback loop associated with *comS*, which encodes its own inducing signal. In the unimodal case the cells import and respond to exogenous XIP, whereas in the bimodal case XIP import is blocked, leaving each cell to respond to its intracellular ComS (or XIP). The first mode allows a generally uniform, population-wide activation of *comX*, but the second mode leads to noisy, positive feedback dynamics in both *comS* and *comX* (Son et al., 2012; Hagen and Son, 2017).

The mechanism of posttranslational control of ComX by MecA/CiCP in *S. mutans* resembles that in pyogenic and salivarius streptococci, to which *S. mutans* MecA is closely homologous (Boutry et al., 2012; Wahl et al., 2014). *S. mutans* MecA is a 240 residue adapter protein that interacts with ComX and CiCP to form a ternary complex that sequesters ComX and targets it for ATP-dependent degradation by the ClpP protease (Tian et al., 2013; Dong et al., 2014). MecA/CiCP similarly controls the master competence regulators ComW in *S. pneumoniae* (Wahl et al., 2014) and ComK in *Bacillus subtilis* (Turgay et al., 1998). In *B. subtilis* MecA was shown to facilitate the ATP-dependent formation of the CiCP proteolytic complex, which unfolds and degrades both MecA and its ComK target, and then itself dissociates (Turgay et al., 1997; Turgay et al., 1998; Kirstein et al., 2006; Mei et al., 2009; Wang et al., 2011; Liu et al., 2013). Therefore MecA/CiCP operates dynamically by continuously turning over MecA as well as its regulatory target if present.

Several studies in *S. mutans* have established that MecA/CiCP suppresses the activation of *comY* under CSP stimulation, in complex media (Tian et al., 2013; Dong et al., 2014; Dufour et al., 2016). Deletion of mecA, *clpC* or *clpP* increased ComX levels and transformability during growth in complex media and also prolonged the competent state. These studies imply that MecA/CiCP serves either to suppress *S. mutans* competence or to switch it off as growth progresses, in complex media. Some studies have found the puzzling result that deletion of *mecA* or *clpCP* caused a weaker increase in ComX levels or transformability – or even had no effect at all – in chemically defined media (with added XIP) than in complex media (with CSP) (Boutry et al., 2012; Tian et al., 2013; Dong et al., 2014; Dufour et al., 2016). A subsequent study found that MecA deletion improved *S. thermophilus* transformability in defined media, although the difference was attenuated at high levels of XIP stimulation (Wahl et al., 2014).

The possible significance of growth media and the presence of heterogeneity raise the question of how MecA/CiCP functions within the full competence pathway, in which the XIP and CSP signaling pathways activate *comX*, or *comY* under CSP stimulation, in complex media, respectively. Although it seems clear that MecA/CiCP inhibits *comY* expression by sequestering and degrading ComX, a clearer model of how this regulation integrates with the known *comX* activation pathway, and how it may be overcome when competence is permitted, is still needed. Additional cell density signals (Dufour et al., 2016), as well as XIP-dependent feedback or additional gene products (Wahl et al., 2014), have been proposed as mechanisms for modulating ComX levels via MecA/CiCP. We have used a single-cell, microfluidic approach to clarify some of these questions and to develop an explicit model of how MecA/CiCP interacts with the noisy and bimodal mechanisms controlling *S. mutans comX*. Our data lead to a simple quantitative model that reproduces both the population average behavior and the cell-to-cell heterogeneity in *comY* activation.
**Results**

**MecA/ClpCP affects XIP-induced transformation of S. mutans**

The transformation efficiencies of the UA159 and mecA/ clpCP deletion strains were measured for cells cultured in defined medium at various concentrations of synthetic XIP and are reported in Table 3 and in Supporting Fig. S1. Overall the deletion of mecA, clpP or clpC did not increase the transformation efficiency at the highest XIP concentrations. This finding is consistent with prior studies showing that deletions of mecA/clpCP do not necessarily enhance streptococcal transformability. Tian et al. (2013) and Dong et al. (2014) reported that the onset of XIP-induced transformability in S. mutans was slightly delayed by deletion of mecA and clpC although these deletions had little effect on the maximum transformation efficiency (Tian et al., 2013; Dong et al., 2014). Similarly in S. thermophilus the deletion of clpC (Børnestad and Havarstein, 2011) or mecA (Boutry et al., 2012) did not increase transformation efficiency in the presence of the ComS peptide. However our data show that XIP concentration modulates the effect of the mecA and clpCP deletions on S. mutans transformability. The transformation efficiencies of the mecA and clpC mutants begin at lower values in comparison to UA159 at low XIP concentrations, but dramatically increase as XIP is increased from 10 to 1000 nM XIP. This is evident in the fold change in transformability for mecA and clpC deletions in particular, as transformation of the mecA strain increased 100-fold and that of clpC increased 20-fold in this range of XIP concentrations. The effect of clpP is less pronounced, but still gives a higher fold change (~7 fold) over these XIP concentrations than does UA159 (~3-fold). Taken together these data support a role for the MecA/ClpCP system in the induction of S. mutans competence by XIP. To obtain more detailed insight into MecA/ClpCP and the control of comY under XIP stimulation we turned to individual cell studies.

**Activation of comX leads to heterogeneous induction of comY**

We used dual fluorescent reporters (PcomX-gfp, PcomY-rfp) to compare the activation of PcomX and PcomY in individual S. mutans supplied with exogenous XIP. Fig. 2A shows S. mutans UA159 growing in microfluidic channels under a constant flow of defined medium (FMC) that contains 0–2 μM XIP. PcomX is activated in all cells if the XIP concentration exceeds about 100 nM, and its activation saturates as XIP exceeds about 800 nM. However, very few cells activate PcomY at XIP concentrations of 400 nM or less, and cells that do activate PcomY vary widely in their red fluorescence intensity. Even at 1–2 μM XIP, many cells exhibit little PcomY activity.

Figurs 2B and C show the statistical distribution of PcomX (GFP, upper rows) and PcomY (RFP, lower rows) reporter fluorescence for cells in response to exogenous XIP or CSP. Reporter fluorescence was imaged while cells grew in microfluidic channels under continuous flow of defined medium for XIP (Fig. 2B), or of complex medium for CSP (Fig. 2C). As previously reported (Son et al., 2012), XIP in defined medium elicits a noisy but generally unimodal (population-wide) comX response. In contrast, CSP in complex medium elicits a much noisier, bimodal (double-peaked distribution) comX response. For both CSP and XIP stimulation, the response of PcomY is highly heterogeneous. Even the highest concentrations of CSP and XIP, which saturate the response of PcomX, incompletely activate PcomY in the population; the PcomY expression levels in individual cells span 2–3 orders of magnitude above the baseline. These data suggest that post-translational regulation of ComX increases cell-to-cell heterogeneity in comY expression, which adds to the noise in the comX response to CSP or XIP stimulation.

The MecA/ClpCP system inhibits the comY response to XIP and increases its noise

To test whether the MecA/ClpCP proteolytic system affects ComX function in defined medium, and to assess its effect on noise in comY expression, we compared comX and comY expression in dual reporter strains in the wild-type (UA159) and ΔmecA genetic backgrounds. Fig. 3 shows PcomY activity in individual cells that were stimulated by XIP in planktonic culture in defined medium and then imaged on glass slides. Similar results were obtained for cells growing in microfluidic flow channels. Deletion of mecA altered the PcomY response in two ways. First, the ΔmecA strain responded more strongly to XIP than did the wild-type. Unlike the wild-type genetic background, the ΔmecA cells showed high median PcomY expression, exceeding the baseline level at XIP concentrations greater than about 200 nM. Second, deletion of mecA reduced noise in comY expression (Fig. 3C and D). The difference in noise is apparent from the magnitude of the variability in the individual cell data, relative to the population median, which is indicated by the horizontal bars in Figs. 3A and C. The ratio of variability to median is clearly larger for UA159 than for ΔmecA. Therefore, although comY and comX expression correlated positively in UA159, the correlation was partially obscured by the noisy behavior of comY. In contrast, comY expression increased systematically as comX expression increased in the ΔmecA strain. Despite some noise in comY, a roughly proportional relationship can be discerned in the data of Fig. 3D,
but not in Fig. 3B. (The upward curvature in Fig. 3D results from the logarithmic horizontal axis.) The nearly linear correlation between $comY$ and $comX$ in the $\Delta$mecA mutant suggests that, in the absence of MecA/ClpCP, ComX activates $comY$ in a direct and predictive fashion.

The effect of MecA on noise in $comY$ is also seen in histograms of $comY$ expression at given $comX$ expression levels. Supporting Fig. S2 shows $comY$ histograms for cells growing in microfluidic channels with flowing defined medium and XIP, binned according to their $comX$ activity. Both at high and low $comX$ activity, the shape of the $comY$ histograms is qualitatively different in the two strains. The deletion of mecA qualitatively alters the relationship between $comY$ and $comX$ expression in defined medium with addition of XIP.
comY and comX expression are simply correlated in the absence of MecA

As is common for bacterial protein expression (Taniguchi et al., 2010), the histograms of PcomY expression (Supporting Fig. S2) resemble a gamma distribution \( \Gamma(n|A,B) \), a two-parameter continuous probability distribution that can be interpreted in terms of sequential, stochastic processes of transcription and translation (see Experimental Procedures). This finding, together with the roughly linear correlation between comY and comX activity in the \( \Delta \text{mecA} \) strain (Fig. 3D), motivates a simple mathematical model for comX/comY in the absence of MecA/ClpCP. The model is described in the Experimental Procedures: comY is activated in a mostly linear (but saturating) fashion by comX on average, but is also subject to stochasticity. The comY activity in a given cell is thus a random variable drawn from a gamma distribution whose parameters are determined by the PcomX activity in the cell. The model has four parameters, which we obtained through a maximum likelihood fit to the \( \Delta \text{mecA} \) individual cell RFP and GFP fluorescence data of Fig. 3D. We then used these parameters to generate a stochastic simulation of the model for comparison to the data.

Fig. 4 compares the \( \Delta \text{mecA} \) experimental data (Fig. 4A and B) with a simulation of the model (Fig. 4C and D). The model accurately reproduces both the population-averaged comY response and its cell-to-cell variability. This result indicates that in the absence of MecA/ClpCP regulation of ComX, comY can be modeled as a typical noisy gene whose average activation is proportional to the concentration of active ComX protein.

A plausible alternative model is that extracellular XIP concentration, rather than PcomX activity per se, controls comY expression in \( \Delta \text{mecA} \). The simulation shown in Supporting Fig. S3 indicates that the best fit of this model significantly overestimates the noise in PcomY. In short, modeling suggests that the PcomX activity of a \( \Delta \text{mecA} \) cell is a straightforward predictor of its PcomY activity, and is also a better predictor than is the XIP concentration.

Different deletions in MecA/ClpCP produce different noise and threshold behaviors in comY

To determine which elements of the MecA/ClpCP system affect sensitivity and noise in comY, we measured PcomY and PcomX activity in the UA159, \( \Delta \text{mecA} \), \( \Delta \text{clpC} \) and \( \Delta \text{clpP} \) genetic backgrounds (Fig. 5). All strains carried the dual fluorescent reporters and were imaged in microfluidic chambers while supplied with a continuous flow of defined medium containing XIP. In all strains, PcomY was more strongly activated at higher XIP concentrations where Pc\text{om}\text{X} expression was higher, although noise and sensitivity varied among the different strains (Fig. 5A). All strains showed a similar dependence of PcomX activity (GFP) on XIP concentration (Fig. 5B). In the relation between comY and comX expression, the UA159 (wild-type) showed a more pronounced threshold in the onset of comY activation, at a comX level near 300 units, and
much greater noise in comY expression. The clpP deletion strain, in which the MecA/ClpC complex can presumably bind, but not degrade, ComX, showed slightly less noisy comY expression than the wild-type and comY was somewhat more readily activated. Deletion of clpC, or especially mecA, reduced comY noise significantly, such that the population was almost uniformly activated when PcomX expression was strong, near 1 μM XIP. Therefore, the interaction between MecA/ClpC and ComX, as well as the proteolytic action of ClpP on that complex, contribute to noise in comY expression and also suppresses the ability of comX expression to elicit the comY response.

Similar data were obtained when cells were grown in static medium and imaged while dispersed on glass slides. The role of MecA alone can be modeled by simple sequestration of ComX

A detailed model for the regulation of ComX by MecA/ClpCP must include the formation of the MecA/ClpC/ComX ternary complex, as well as the kinetics of ComX and MecA degradation by ClpP. Both of these mechanisms are absent in the ΔclpC strain, although the binary interaction of MecA with ComX is present. Therefore, we tested whether a binary sequestration (MecA + ComX) model could reproduce our data for the activation of comY by ComX in the ΔmecA strain. In this model, described in Experimental Procedures, individual ComX molecules are presumed to be tightly sequestered by individual MecA molecules, leaving them unavailable to stimulate comY transcription. Then the probability distribution for the comY expression of a cell becomes determined not by its comX activity alone, but by the excess of ComX over MecA copy numbers. We modeled the MecA copy number as a random variable drawn from a gamma probability distribution; the activation of comY by the available (unsequestered) ComX is modeled as in Fig. 4. The MecA probability distribution is presumed to be independent of XIP, consistent with our mRNA measurements showing no effect of XIP on mecA, clpC or clpP expression (Supporting Table ST1). Fitting this MecA model to the ΔclpC data therefore requires only a two-parameter fit for the gamma distribution parameters, which we obtained by maximum likelihood comparison of the data and model.

Fig. 6 compares the ΔclpC data with a stochastic simulation of this model. The comY–comX correlation closely resembles the experimental data, both in its average...
Thresholding and stochasticity from MecA/ClpCP

Fig. 5. A. Effect of mecA/clpCP deletions on the correlation between comY and comX activation. For each of the four strains (UA159, ΔmecA, ΔclpP, ΔclpC) each point shows the PcomY and PcomX activity of one cell, as measured by RFP and GFP reporters, respectively. Cells were imaged while growing in microfluidic channels that were supplied with a continuous flow of defined medium that contained XIP concentrations as indicated by the point color. Approximately 1000 cells of each strain were imaged at each XIP concentration. Solid lines indicate the 10, 30, 50, 70 and 90th percentiles of PcomY activity. Cell autofluorescence contributes a background red fluorescence that is typically 1–5 fluorescence units in most experiments. Cell autofluorescence contributes a green background that is typically 20–30 fluorescence units. Supporting Fig. S6 shows the same data on linear axes.

B-C Scatterplots showing individual cell comX and comY expression versus exogenously added XIP in the four strains. [Colour figure can be viewed at wileyonlinelibrary.com]
trend and its noise. These results show that the higher comY expression noise that is observed in the ΔclpC strain, compared to the ΔmecA strain, is consistent with a mechanism where MecA suppresses comY response by sequestering ComX. Fitting the model to the data provides the probability distribution of the MecA copy number, Fig. 6C, where MecA is measured in units of equivalent PcomX activity. Cell-to-cell variability in MecA copy number is then a source of variability in comY expression.

CSP and XIP stimulation produce similar correlations between comX and comY activation

Previous studies have demonstrated that deletions of mecA or clpCP enhance comY expression upon stimulation with CSP in complex media (Tian et al., 2013; Dong et al., 2014). Our data show with single-cell resolution that the same deletions also affect the response to XIP in defined media. These findings raise the question of whether, in the presence of MecA/ClpCP, the activation of comY by ComX may be similar regardless of how comX transcription is induced, whether by XIP or CSP. Fig. 7 compares single-cell measurements of comX and comY activity with CSP and XIP, respectively. Precise quantitative comparison of the two response curves is complicated by the stronger green auto-fluorescence of cells in complex medium, which shifts the horizontal axis of the CSP data. Further, CSP appears to induce a slightly noisier comY response than does XIP, possibly in connection with feedback behavior in the ComDE system (Son et al., 2015). However, the data verify a generally similar behavior in both conditions: comY responds in threshold fashion to activation of comX, and comY activation is highly heterogeneous in the population, even among cells with the highest comX activity.

Discussion

The MecA/ClpCP proteolytic system is well conserved as a negative regulator of genetic competence across streptococcal groups and in other naturally competent species, including B. subtilis (Liu et al., 2013). However, while mechanistic studies of MecA/ClpCP have provided a clear description of its action, they have not fully resolved the question of how MecA/ClpCP contributes to competence regulation. Several authors have proposed that MecA/ClpCP serves either to suppress or terminate the competent state. For B. subtilis, Turgay et al. proposed that MecA/ClpCP degradation of the ComK competence regulator provides a ‘timing’ function by limiting synthesis of the auto-activating ComK regulator, thus permitting escape from the competent state (Turgay et al., 1998). Dufour et al. proposed a similar model for S. mutans, in which the sequestration and degradation of free ComX by MecA/ClpCP forces an exit from the competent state late in growth, when the transcription of comX is repressed (Dufour et al., 2016). Wahl et al. proposed that S. thermophilus MecA/ClpCP serves a ‘locking’ function, preventing the cell from entering the competent state under inappropriate conditions, such as early in the growth phase (Wahl et al., 2014). Wahl et al. argued that at low XIP concentrations proteolytic degradation of ComX prevents competence, but that high XIP concentrations may alleviate this repression, possibly by overwhelming the proteolytic capacity or by activating another, unidentified gene product.
Both the ‘locking’ and ‘timing’ models interpret MecA/ClpCP as a mechanism for suppressing activation of comY when comX expression is weak. Our data are consistent with this description. Moreover, our data show that this suppression can be described by the simplest model in which an intracellular pool of MecA intercepts available ComX, sequestering it and blocking its otherwise straightforward activation of comY. Such a model quantitatively fits the data on the clpC mutant, in which MecA can sequester ComX but clpP proteolysis is absent. If the MecA copy number obeys a gamma probability distribution, as is common for bacterial proteins, then the model reproduces both the average relationship between comY and comX expression and the cell-to-cell variability in that expression. Therefore, the response of comY in individual clpC and mecA cells can be understood solely in terms of the PcomX activity and MecA copy number distribution. The behavior of the late competence genes in these mutants can be understood without positing any role for XIP other than as a stimulus for PcomX.

In addition, our single-cell data show that the MecA/ClpCP system substantially enhances the noise (cell-to-cell heterogeneity) in comY expression when comX is activated. Even at high XIP concentrations that saturate comY expression, comX expression levels within the UA159 population span a range extending three orders of magnitude above the baseline; by contrast, the deletion mutants all express comY with far less noise at high XIP concentrations. Our modeling indicates that cell-to-cell variability in the MecA copy number in wild-type cells, together with the proteolytic action of ClpP (which reduces MecA and ComX copy numbers) adds to noise that is generated upstream by the pathways that activate comX. The resulting noisy threshold effect is very similar to the toxin/antitoxin competition that generates phenotypic heterogeneity in bacterial persistence (Rotem et al., 2010), or to a sequestration-induced threshold model for nonlinear gene regulation (Buchler and Cross, 2009).

A clear understanding of the role of MecA/ClpCP has perhaps been complicated by early reports that deletion of mecA or clpC increased transformability or ComX protein levels under CSP stimulation (in complex medium), but not under XIP stimulation (in defined medium). Our data confirm in detail that the MecA/ClpCP system affects signaling from comX to comY in defined medium. In fact, as the sequestration model described above is indifferent to whether comX is stimulated by exogenous XIP or CSP, we expect that signaling from comX to comY should be similar in both CSP/complex medium and in XIP/defined medium. Fig. 7 suggests that the relationship is very similar.

This finding suggests that the MecA/ClpCP system acts continuously to suppress ComX levels, regardless of the extracellular inputs driving comX expression. A model where MecA/ClpCP performs this task in relatively steady fashion is consistent with findings that S. mutans MecA and ClpCP protein levels did not differ in complex and defined medium (Dong et al., 2014), that MecA induction showed little change during S. suis competence (Zaccaria et al., 2016), and that S. mutans mecA/clpCP mRNA levels are insensitive to XIP inputs (Supporting Table ST1). Thus competence will be suppressed when comX is weakly expressed due to insufficient CSP or XIP early in growth (‘locking’ behavior). Competence will also be suppressed when comX is weakly expressed late in growth due to inefficient CSP/XIP signaling. Falling extracellular...
pH late in the growth phase suppresses competence signaling by CSP and XIP (Guo et al., 2014; Son et al., 2015), which may allow MecA/ClpCP to shut down the competent state (‘timing behavior’).

Consequently the sequestration mechanism can provide both ‘timing’ and ‘locking’ functions. The simulations in Fig. 4 and Fig. 6 are based on simple equilibrium models that address only the effects of sequestration by MecA on the pool of free ComX, omitting the kinetic effects of ClpCP unfolding and degradation of ComX and MecA. A model that includes ClpP proteolysis is much more complicated, as it must include the sequential binding steps that are associated with the formation of the ternary complex, binding of ClpP, and the breakdown of both MecA and ComX. The binding and kinetic parameters of the model cannot be determined from our data; however we can construct a reasonably tractable model for the full system by simplifying the complex regulatory mechanism that is outlined in the literature (Mei et al., 2009). Supporting Fig. S7 describes a simplified kinetic model that can rationalize some of the observations in our data, including the finding that deletion of clpC or clpP did not eliminate the comX threshold that is required for comY activation, and that only the mecA deletion eliminated the threshold and sharply reduced the noise in comY. Supporting Fig. S7 shows that simulations from such rough models can reproduce key differences in comX-comY threshold behavior observed among the mutants studied here.

We note that a MecA copy number distribution that has higher mean but is narrower than that of Fig. 6C would still provide the same ‘timing’ or ‘locking’ function without introducing as much noise in comY. The evident width of the distribution therefore suggests that the organism may benefit from greater noise. The competence pathway in S. mutans is linked to several stress-induced behaviors that are heterogeneously distributed in the population, including competence, lysis and a persister phenotype (Perry et al., 2009; Leung et al., 2015; Leung et al., 2015). A link between quorum controlled behavior and phenotypic heterogeneity has often been noted in bacterial gene regulation. In other organisms, such as B. subtilis, complex pathways that integrate intracellular and extracellular signaling mechanisms with stochastic gene expression often generate phenotypic heterogeneity, distributing stress response behaviors such as competence and sporulation among different individuals in the population (Smits et al., 2006; Dubnau and Losick, 2006; Veening et al., 2008; Grote et al., 2015). Interestingly, propidium iodide staining of individual S. mutans indicates that comX-driven lysis is decoupled from comX-driven competence (Supporting Fig. S5). While higher comX expression increases the probability of cell lysis, the most highly expressing cells (which are more likely to express comY) actually show less evidence of lysis. Accordingly, the MecA/ClpCP system may provide a bet-hedging advantage to an S. mutans population by providing an additional, stochastic switching point in the regulatory pathway from stress conditions to transformability.

Our data show that the action of the S. mutans MecA/ClpCP system can be quantitatively understood, at the level of individual cell behavior, within a very simple threshold mechanism. As the MecA/ClpCP system is widely conserved this finding raises the question of whether MecA/ClpCP also generates a heterogeneity advantage in other organisms such as S. pneumoniae, in which competence regulation is more straightforward and the comX bimodality mechanism is absent. Our data also highlight the long-standing question of whether by combining cooperative behaviors of quorum signaling with deliberately noisy intracellular phenomena such as MecA and ComRS, S. mutans can achieve some form of optimum balance between socially driven, environmentally driven and purely stochastic behavior in competence regulation.

Experimental procedures

Preparation of reporter strains

S. mutans strains and deletion mutants (Table 1) harboring green fluorescent protein (gfp) and/or red fluorescent protein (rfp) reporter genes fused to the promoter regions of comX (PcomX-gfp ) and comY (PcomYA-rfp) were grown in brain heart infusion medium (BHI; Difco) at 37°C in a 5% CO2, aerobic atmosphere with either spectinomycin (1 mg mL−1), erythromycin (10 μg mL−1) or kanamycin (1 mg mL−1). PcomX-gfp was directly integrated into the chromosome of S. mutans (denoted XG) by amplifying a 0.2 kbp region comprising PcomX using primers that incorporated Xbal and SpeI sites (Table 2). This was fused to a gfp gene that had been amplified with primers engineered to contain SpeI and Xbal sites from the plasmid pCM11 (Lauderdale et al., 2010; Son et al., 2012), and inserted into the Xbal site on pBGE (Zeng and Burne, 2009). PcomYA-rfp was constructed in shuttle vector pDL278 (LeBlanc et al., 1992) by amplification of a 0.2 kbp region containing PcomY with HindIII and SpeI site-containing primers and fusing with the DsRed.T3(DNT) rfp gene reporter fragment amplified from plasmid pRFP (Bose et al., 2013), using primers that incorporated SpeI and EcoRI sites. The ligation mixtures were transformed into competent S. mutans (strain designated YR) and into the XG strain (denoted XG&YR). Additionally, to study the role of MecA/ClpCP on PcomY expression, both the XG integration vector and the YR shuttle vector were transformed into strains harboring non-polar (NPKmR) antibiotic resistance cassette
replacements of mecA (this study), clpC or clpP (Lemos and Burne, 2002). Plasmid DNA was isolated from Escherichia coli using a QIAGEN (Chatsworth, Calif.) Plasmid Miniprep Kit. Restriction and DNA-modifying enzymes were obtained from Invitrogen (Gaithersburg, Md.) or New England Biolabs (Beverly, Mass.). PCRs were carried out with 100 ng of chromosomal DNA using Taq DNA polymerase. PCR products were purified with the QIAquick kit (QIAGEN). DNA was introduced into S. mutans by natural transformation and into E. coli by the calcium chloride method (Cosloy and Oishi, 1973).

**Competence peptides**

Synthetic CSP (sCSP, sequence = SGSLSTFFRLFNRS-FTQA), corresponding to the mature 18 residue peptide (Hossain and Biswas, 2012) was synthesized by the Interdisciplinary Center for Biotechnology Research (ICBR) facility at the University of Florida and its purity (95%) was confirmed by high-performance liquid chromatography (HPLC). The CSP was reconstituted in water to a final concentration of 2 mM and stored in 100 μL aliquots at −20°C. Synthetic XIP (sXIP, sequence GLDWWSL), corresponding to residues 11–17 of ComS, was synthesized and purified to 96% homogeneity by NeoBioSci (Cambridge, MA). The lyophilized XIP was reconstituted with 99.7% dimethyl sulfoxide (DMSO) to a final concentration of 2 mM and stored in 100 μL aliquots at −20°C.

**Microfluidic mixer design and fabrication**

Microfluidic devices were fabricated by the soft lithography method of molding a transparent silicon elastomer (polydimethylsiloxane) on a silicon master (Sia and Whitesides, 2003). The master was made from a silicon wafer through conventional photolithographic processing. Details of the fabrication method and the devices were described previously (Jeon et al., 2000; Son et al., 2012; Son et al., 2015). Briefly, the microfluidic device consisted of nine parallel flow chambers (each 15 μm deep and 400 μm wide). Three inlet channels supplied media containing different concentrations of signal peptides, delivered by syringe pumps into the device. The design has a mixing network that generates nine streams containing different admixtures of the three input solutions. These streams flow through the nine cell chambers in which S. mutans are adhered to the lower, glass window. The device also has two side channels: one for the control of fluid inside the device and the other for injection of different solutions into the cell chambers. Two-layer lithography allows air pressure control of these side channels during cell loading and injection of different solutions (Unger et al., 2000).

**Microfluidic experiments**

Overnight cultures grown in BHI with antibiotic selection were washed and diluted 20-fold in fresh medium, which was either chemically defined medium (FMC) (Terleckyj et al., 1975; De Furio et al., 2017) or a complex medium that consisted of 1/3 of BHI (BD) and 2/3 of FMC by volume. Cultures were then incubated at 37°C in a 5% CO₂, aerobic atmosphere. When OD₆₀₀ reached 0.1–0.2, cells were sonicated at 30% amplitude for 10 s (Fisher FB120) to separate cell chains and then loaded into the microfluidic device. Each cell chamber was continuously perfused with fresh medium containing different amounts of synthetic XIP (0–2 μM) or synthetic CSP (0–1 μM). The XIP or CSP concentration in each flow channel was generated by the mixture of three different input media in the mixing network in the device. A trace amount (0–10 ng/mL) of far-red fluorescent dye (Alexa Fluor 647) was added to each of the three inlet media in proportion to its signal molecule concentration, so that the concentration of signal molecule in each chamber could be calculated. After 2.5 hr of incubation time, fresh medium containing 100 μg mL⁻¹ of rifampicin was flowed through all cell chambers to halt GFP and RFP transcription. Cell chambers were then incubated an additional 3 hr to allow the full maturation of RFP. Cells were imaged in phase contrast and in green and red fluorescence using an inverted microscope (Nikon TE2000U) equipped with a computer controlled motorized stage and cooled CCD camera. Approximately 700 to 1000 cells were studied for each condition shown in the histograms. The microfluidic study of comY and comX response to XIP in the mutant strains was repeated three times. The same experiment was also performed on cells that were dispersed on

### Table 1. Strains used in this study.

| S. mutans strains | Characteristic(s) | Source |
|------------------|-------------------|--------|
| WT (UA159)       | S. mutans wild-type strain | ATCC 700610 |
| XG               | P₅’_comXᵗ’_rfp integrated into UA159, Em’ | This study |
| YR               | UA159 harboring P₅’_comXᵗ’_rfp, Em’ | This study |
| XG&YR            | XG harboring P₅’_comXᵗ’_rfp, Sp’ | This study |
| XG&YR & ΔmecA    | ΔmecA::NPKm’ into XG&YR | This study |
| XG&YR & ΔclpC    | ΔclpC::NPKm’ into XG&YR | This study |
| XG&YR & ΔclpP    | ΔclpP::NPKm’ into XG&YR | This study |
glass slides, giving closely similar results. Similarly the microfluidic study of comY and comX response to CSP stimulation in UA159 was repeated three times.

**Single cell image analysis**

Custom Matlab software was used to analyze the expression of the gfp and rfp reporters in individual cells from overlaid phase contrast, GFP and RFP images (Kwak et al., 2012). The software first segments individual cells from the cell chain based on the phase contrast image, then finds the concentration of GFP and RFP by correlating the intensity of the phase contrast image with its GFP and RFP fluorescence intensity. This gives a unitless parameter (denoted \( R \)) that is proportional to the intracellular concentration of GFP or RFP. The GFP or RFP expression levels shown in the data figures are the \( R \)-values for green or red cell fluorescence respectively.

**Transformation efficiency**

Overnight cultures of selected strains were grown overnight in BHI medium with appropriate antibiotic (\( n = 8 \) replicates). The next day, cultures were diluted 1:20 in 200 \( \mu \)L of FMC medium in polystyrene microtiter plates and grown to OD\(_{600}\) = 0.15 in a 5% CO\(_2\) atmosphere. At this time, different synthetic XIP concentrations (10–1000 nM) and 0.5 \( \mu \)g of purified plasmid pIB184, which harbors an erythromycin resistance (ErmR) gene, were added to the cultures. After 2.5 hr incubation at 37°C, transformants and total CFU were enumerated by plating appropriate dilutions on BHI agar plates with and without the addition of 1 mg mL\(^{-1}\) erythromycin, respectively. CFU were counted after 48 hr of incubation, and transformation efficiency was expressed as the percentage of transformants among the total viable cells.

**RFP expression and stability**

To verify that the codon-optimized variant of DsRed.T3 rfp reporter produces similar results to gfp, UA159 harboring PcomX-rfp plasmid was grown in a static BHI or FMC medium. When the OD\(_{600}\) reached \( \sim 0.2 \), each culture was divided into two samples, with and without the signaling peptide, respectively. 1 \( \mu \)M synthetic CSP was the signal for the BHI sample and 500 nM synthetic XIP was the signal for the FMC sample. After 2 hours of stimulation, rifampicin was added. Samples were incubated an additional 3 hr for RFP maturation, and their RFP expression was measured. Supporting Fig. S4A shows that the RFP fluorescence successfully captured the distinct bimodal (CSP) and unimodal (XIP) response of comX under these conditions.

To test the maturation time of the RFP protein (Supporting Fig. S4B), UA159 harboring PcomX-gfp and PcomY-rfp was grown in static FMC medium. When the

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**Table 2. Primers used in this study. Underline of nucleotide sequence denotes respective restriction enzyme site.**

| Primer           | Nucleotide Sequence (5’ – 3’)                  |
|------------------|------------------------------------------------|
| PcomX-XbaI-FW    | GGA TCT AGA CCA ATT TCA AAT AAT G              |
| PcomX-SpeI-RV    | CTT CAC TAG TCT ATT ACG ATG ACC                |
| PcomY-HindIII-FW | ACA AAG GGG AAA CAA AAT GAT ACC C              |
| PcomY-SpeI-RV    | TCG ACT AGT CCA GGA AAA AAT TAG                |
| rfp-SpeI-FW      | GAC TAG TTG ATT AAC TTT ATA AGG AGG AAA AAT ATA TGG A |
| rfp-EcoRI-RV     | CGG AAT TCT TAT AAA AAC AAA TGA TGA CGA CCT TCT GTA C |
| ΔmecA-FW         | GAT GAC TGG CTG GAT GCA CA                     |
| ΔmecA-BamHI-FW   | TTT GGA TCC CAT AGT TCT TAC CTC A              |
| ΔmecA-BamHI-RV   | ATG GGA TCC TAA GCT AGA TGA TAC C              |
| ΔmecA-RV         | CCA AAC CAT CCA AAC CAT CAA                    |

**Table 3. Transformation efficiencies measured in defined medium.**

| Synthetic XIP (nM) | UA159       | ΔmecA       | Δ clpC       | ΔclpP       |
|-------------------|-------------|-------------|-------------|-------------|
| 10                | 3.26 ± 0.99 e-5 | 6.23 ± 2.01 e-7 | 5.30 ± 2.74 e-7 | 6.31 ± 1.45 e-6 |
| 25                | 5.24 ± 1.40 e-5 | 1.35 ± 0.54 e-5 | 1.15 ± 0.37 e-6 | 8.47 ± 2.22 e-6 |
| 50                | 5.95 ± 2.34 e-5 | 3.14 ± 0.84 e-5 | 2.04 ± 0.85 e-6 | 8.81 ± 1.97 e-6 |
| 75                | 4.85 ± 0.98 e-5 | 4.21 ± 1.40 e-5 | 2.43 ± 0.73 e-6 | 9.21 ± 2.73 e-6 |
| 100               | 5.51 ± 3.59 e-5 | 7.92 ± 1.08 e-5 | 5.49 ± 1.32 e-6 | 1.86 ± 0.78 e-5 |
| 300               | 9.07 ± 3.11 e-5 | 1.02 ± 0.42 e-4 | 1.11 ± 0.20 e-5 | 3.57 ± 1.03 e-5 |
| 1000              | 9.65 ± 2.59 e-5 | 5.78 ± 0.55 e-5 | 1.19 ± 0.20 e-5 | 4.34 ± 2.91 e-5 |
OD_600 reached ~ 0.2, 2 μM synthetic XIP was added to induce RFP production. After additional incubation of 2 hr, 100 μg/mL of rifampicin was added. Then RFP fluorescence was measured every hour up to 5 hr. Supporting Fig. S4B shows that fluorescence saturated between 2 and 3 hr, suggesting 3 hr is sufficient waiting time for RFP maturation. The corresponding half-time of ~1.4 hr is in good agreement with the reported DsRed. T3 maturation half time of ~1.3 at 37 °C (Bevis and Glick, 2002).

To test the expression and stability of the RFP protein (Supporting Fig. S4C and S4D) in the different mutant backgrounds used in this study, the RFP gene was transcriptionally linked to the constitutive promoter (Pveg) (Radeck et al., 2013) by cloning into HindIII and SpeI sites on the pDL278 plasmid. Following ligation, the selected construct was then transformed into each of the UA159, ΔmecA, ΔclpP and ΔclpC backgrounds with colonies selected by spectinomycin resistance and confirmed by sequencing. After a 1:25 dilution from overnight cultures, cells were grown to late mid-exponential growth phase (OD_600_mn = 0.8) in BHI medium. A final concentration of 35 μg/mL chloramphenicol was then added to the cultures to halt protein translation (t = 0 hr). A fraction of the culture was harvested at selected time points after chloramphenicol addition (0, 1, 3 and 6 hr). Whole cell lysates from each fraction were obtained by mechanical disruption (bead beating) and 10 μg was loaded and run on a 4–20% gradient Mini-PROTEAN TGX gel (BioRad). Following transfer to a polyvinylidene difluoride (PVDF) membrane (BioRad), the RFP protein was probed for with a Living Colors DsRed Polyclonal Antibody (Takara Bio USA, (BioRad), the RFP protein was probed for with a Living Colors DsRed Polyclonal Antibody (Takara Bio USA, Inc) according to the supplier’s protocol.

For the single-cell fluorescence measurements of Supporting Fig. S4D, cultures of the deletion and wild-type strains were grown in BHI overnight with 1 mg/mL spectinomycin. Cultures were then washed twice in FMC, diluted 20-fold and ultrasonicated before being resuspended in FMC. Cultures were then incubated at 37°C to OD_600 = 0.1. Aliquots were then removed at hourly intervals, dispersed on glass slides, and the cells imaged in phase contrast and fluorescence. Fluorescence data shown were collected at 3 hr.

**mRNA levels for mecA, clpCP and com genes**

Data for the analysis of relative mRNA levels for mecA, clpCP and com genes are from RNA-Seq analysis completed on strain UA159 (Kaspar et al., 2018, in preparation). The wild-type strain was grown in FMC medium to OD_600 = 0.2, at which time either a final concentration of 2 M XIP or vehicle control (1% DMSO) was added. The strains were then allowed to grow to mid-exponential phase (OD_600 = 0.5) before harvesting. From the analyzed RNA-Seq data, total read counts for each selected gene were found from three biological replicates and RPKM (reads per kilobase per million) calculated under each condition. Finally, ratios for mRNA levels were found using the normalized RPKM data and by setting mecA levels to 1.0. The data files used in this study are available from NCBI-GEO (Gene Expression Omnibus) under accession no. GSE110167.

**Stochastic model for MecA regulation of comX**

We used the gamma statistical distribution to model cell-to-cell variability (noise) in the activation of comY by ComX and the effect of the MecA/ClpCP system. Heterogeneity in bacterial protein copy number can be well described by a physical model of transcription and translation as consecutive stochastic (Poisson) processes, characterized by rates k_{g} (transcripts per unit time) and k_{p} (protein copies per transcript per unit time), respectively (Friedman et al., 2006; Taniguchi et al., 2010). In this model, the protein copy number n in each cell is a random variable drawn from a gamma distribution \( \Gamma(n \mid A, B) \). The two parameters A and B that determine the shape of the distribution are related to \( k_{g} \) and \( k_{p} \), respectively (and to the mRNA and protein lifetimes) (Friedman et al., 2006). Gamma distribution fits to our PcomY reporter data are shown in Supporting Fig. S2.

To model ComX activation of comY in the mecA deletion mutant (lacking post-translational regulation by MecA/ClpCP), we applied a simple quantitative model in which the PcomX activity of each cell, as reported by GFP fluorescence, determines the gamma distribution for its PcomY activity, measured by RFP fluorescence. Specifically, the PcomY-rfp reporter fluorescence \( Y \) of a cell is a random number drawn from a gamma distribution \( \Gamma(Y \mid A, B) \), for which the parameters are
\[
A = a_1 X/(X + a_2) \\
B = b_1 X/(X + b_2)
\]

Here, \( X \) is the PcomX-gfp reporter fluorescence of that cell. Thus \( Y \) is directly activated by \( X \) in a saturating but noisy fashion. We fit this model to a dataset of individual cell RFP and GFP fluorescence values collected on dual reporter (PcomX-gfp, PcomY-rfp) \( \Delta \)mecA cells that were supplied with different concentrations of synthetic XIP (defined medium) and then imaged on glass slides. Maximum likelihood analysis gives the four model parameters \( a_1, a_2, b_1, b_2 \) for the \( \Delta \)mecA strain as follows: we start with the experimental PcomX activity measured for each cell, then use the four parameters to define a PcomY gamma distribution for that cell. We find the probability of
that cell’s actual PcomY activity, given that gamma distribution. The parameter values are then adjusted to maximize the likelihood of the total dataset. (The optimal values are given in Supporting Fig. S3.) Given these model parameters we then generate a model simulation for comparison against the data as follows: we use the model parameters and the measured PcomX activity of each cell to generate its PcomY gamma distribution, draw a random number from that distribution to obtain a simulated PcomY activity for the cell, and then plot the resulting simulated PcomY vs PcomX values for all cells (Fig. 4C, 4D).

We also tested an alternative model in which environmental XIP concentration, rather than PcomX activity of a cell, is the determinant of that cell’s PcomY activity. In this model, X in the above equations refers to the XIP concentration supplied to a cell. Again, using maximum likelihood, we found the parameters \((a_0, a_x, b_0, b_x)\) that gave best agreement with the \(\Delta m\)eCA data in this alternative model. The scatterplot of Supporting Fig. S3C, generated by the above simulation procedure, compares the simulated PcomY to the experimental PcomY for the \(\Delta m\)eCA data.

For the dual-reporter \(\Delta clpC\) mutant, we extended the above model by allowing MecA to sequester, but not degrade ComX. For simplicity, we assume that (i) MecA and ComX bind with sufficiently high affinity that a cell can only activate \(\text{com}Y\) to the extent that its ComX copy number exceeds its number of MecA copies, leaving some available ComX; (ii) The activation of \(\text{com}Y\) by the available ComX is as described in the \(\Delta m\)eCA model above (and with the same parameters); (iii) the MecA copy number \(M\) in a cell is a stochastic variable drawn from a gamma distribution \(\Gamma(M \mid A, B)\) whose \(A\) and \(B\) parameters are fixed, independent of XIP concentration. If \(X\) is the PcomX-gfp activity of a given cell, then \(X' = X - M\) is the amount of ComX available after sequestration by MecA. Given a GFP measurement of \(X\) for a cell, the MecA gamma distribution \(\Gamma(M \mid X', A, B)\) determines the probability that \(X'\) copies of ComX are available to activate \(\text{com}Y\). This \(X'\) determines the probability distribution for \(Y\) (the PcomY-rfp response) by the above model. Averaging over the MecA distribution then gives a prediction for both the average behavior and cell-to-cell variability in the dependence of PcomY-rfp on PcomX-gfp, in the presence of MecA.

Taking the PcomY-rfp activation parameters obtained in the \(\Delta m\)eCA fit, we therefore analyzed individual cell PcomX/PcomY data that was collected on \(\Delta clpC\) cells that were supplied with different XIP concentrations and imaged on glass slides. We then found the \(A\) and \(B\) values for the MecA distribution that maximize the likelihood of the PcomX/PcomY dataset, given the sequestration model. Using those parameters, we then generated a simulation of the PcomY versus PcomX activity. We compared these results to the experimental data for the \(\Delta clpC\) strain.

In plotting the simulation (Fig. 6), we modeled the weak red auto-fluorescence background in the data by adding baseline Gaussian noise of \(3 \pm 0.8\) red fluorescence units; this baseline is small compared to the typical red fluorescence (~\(10^2 – 10^4\) units) of \(\text{com}Y\) activated cells.

### Acknowledgements

The authors thank Momin Haider for collecting the data in Supporting Fig. S4D. This work was supported by 1R01 DE023339, DE13239 and T90 DE021990 from the National Institute of Dental and Craniofacial Research.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.