Quorum sensing integrates environmental cues, cell density and cell history to control bacterial competence

Stefany Moreno-Gámez[1,2], Robin A. Sorg[1], Arnau Domenech[1,4], Morten Kjos[1,3], Franz J. Weissing[2], G. Sander van Doorn[2,5] and Jan-Willem Veening[1,4,5]

1. Molecular Genetics Group, Groningen Biomolecular Sciences and Biotechnology Institute, Centre for Synthetic Biology, University of Groningen, Nijenborgh 7, 9747 AG Groningen, the Netherlands

2. Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, P.O. Box 11103, 9700 CC, The Netherlands

3. Present address: Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, N-1432 Ås, Norway

4. Present address: Department of Fundamental Microbiology, Faculty of Biology and Medicine, University of Lausanne, Biophore Building, CH-1015 Lausanne, Switzerland

5. Correspondence to G. Sander van Doorn: g.s.van.doorn@rug.nl, tel: +31 (0)50 363 8097 and Jan-Willem Veening: Jan-Willem.Veening@unil.ch, tel: +41 (0)21 6925625, Twitter: @JWVeening

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Abstract

Streptococcus pneumoniae becomes competent for genetic transformation when exposed to an autoinducer peptide named CSP. This peptide was originally described as a quorum-sensing (QS) signal, enabling individual cells to regulate competence in response to population density. However, recent studies suggest that CSP may instead serve as a probe for sensing environmental cues, such as antibiotic stress or environmental diffusion. Here, we show that competence induction can be simultaneously influenced by cell density, external pH, antibiotic-induced stress and cell history. Our experimental data is explained by a mathematical model where the environment and cell history modify how cells produce or sense CSP. Taken together, model and experiments indicate that autoinducer concentration can function as an indicator of cell density across environmental conditions, while also incorporating information on environmental factors or cell history, allowing cells to integrate cues such as antibiotic stress into their QS response. This unifying perspective may apply to other debated QS systems.

Introduction

Bacteria release small diffusible molecules in the extracellular medium known as autoinducers. These molecules induce the expression of particular functions including biofilm formation, luminescence and genetic competence as well as their own production [1,2]. The most prevalent functional interpretation of the production and response to autoinducers is known as quorum sensing (QS). According to this view, the concentration of autoinducer molecules is a proxy for cell density, allowing bacteria to regulate the expression of those phenotypes that are only beneficial when

expressed by many cells [1,2]. However, it is likely that the concentration of autoinducer molecules does not only reflect cell density, but that it is also affected by environmental factors, such as the diffusivity of the medium. In fact, alternative hypotheses state that bacteria release autoinducers to sense these environmental factors rather than to monitor cell density. The most well-known hypothesis proposed by Redfield is that the function of autoinducers is diffusion sensing, allowing cells to avoid the secretion of costly molecules under conditions where they would quickly diffuse away [3]. Other potential roles suggested for autoinducer production are sensing local cell density together with diffusion [4], the positioning of other cells during biofilm formation [5] and temporal variations in pH [6].

We study pneumococcal competence, a system classically used as an example of QS. However, whether competence is actually controlled by QS has been recently debated. Competence is a transient physiological state that is developed by *Streptococcus pneumoniae*, as well as other bacteria. Upon entry into competence, pneumococci upregulate the expression of genes required for uptake of exogenous DNA as well as bacteriocins and various genes involved in stress response [7]. In *S. pneumoniae*, competence is regulated by an autoinducer molecule known as the competence-stimulating peptide (CSP) in a two-component regulatory system formed by the histidine kinase ComD and the response regulator ComE [8,9] (Figure 1). Despite the detailed understanding of the regulatory network of competence induction, little is known about why competence is controlled by an autoinducer peptide like CSP. CSP has been classically thought to be a QS signal[10], whose function could be to monitor the density of potential DNA donors[14]. However, competence can be induced in response to environmental factors like pH, oxygen, phosphate and antibiotic stress [11–14]. Based on this evidence and the finding that competence initiates at the same time in

pneumococcal cultures inoculated at different initial densities, it was suggested that CSP acts as a timing device that allows cells to mount a timed response to environmental stress independently of cell density [15,16]. Since then, this hypothesis has established in the field as an alternative to the QS view of competence [13,16–18]. Recently, Prudhomme et al. renamed the timing device mechanism as a growth-time dependent mechanism and proposed that a subpopulation of competent cells that originates stochastically spreads the competent state to the rest of the population by cell-cell contact [19]. Another alternative to QS is that pneumococcal competence is an instance of diffusion sensing. This was suggested by Yang et al. based on the observation that the quorum for competence induction is not fixed but decreases with more restrictive diffusion [20].

Here, we study the regulation of pneumococcal competence by cell density and two environmental factors, antibiotic stress and pH. Using batch-culture experiments, single cell analyses and mathematical modeling we show that these factors simultaneously regulate competence development because they all affect the CSP concentration: cell density sets the amount of cells producing CSP, whereas the environment and cell history modify the rate at which individual cells produce or sense CSP. Since there is density regulation and we show that CSP is exported extracellularly, we advocate to keep using the term ‘quorum sensing’ in the context of pneumococcal competence but with a broader meaning to acknowledge that in addition to cell density, multiple factors are integrated into this QS response.

Results

A mathematical model of pneumococcal competence development

We developed a mathematical model of pneumococcal competence based on the network of protein interactions known to regulate competence development (Figure 1) during growth in a well-mixed liquid medium. Briefly, the precursor of CSP, ComC, is cleaved and exported to the extracellular space by the membrane protein complex ComAB [9,21]. Upon binding to CSP, ComD phosphorylates the response regulator ComE, which in its phosphorylated form upregulates transcription of the operons *comAB*, *comCDE* and *comX* [9,22,23]. The latter encodes the sigma factor ComX, which controls transcription of genes required for uptake and processing of exogenous DNA [7,24]. Our model uses ordinary differential equations (ODEs) and consists of two components. At the population-level it keeps track of the population density and the extracellular concentration of CSP; at the cell-level it keeps track of the intracellular concentrations of the proteins involved in competence regulation (Figure 1). All the cells export CSP to the medium at a rate determined by the intracellular concentrations of ComC and ComAB. The concentration of CSP then feeds back into the intracellular concentrations of all the proteins involved in competence since their transcription rates depend on the ratio of ComE to ComE~P and thus on the rate at which ComD phosphorylates ComE. Different environmental scenarios are simulated by changing model parameters according to the known effects of such environmental factors in the competence regulatory network. Since the model is primarily concerned with competence initiation we purposely left out genes crucial for other aspects of competence development (e.g. the stabilizing factor ComW and the immunity gene *comM*) [17] and genes involved in competence shut-off such as DprA [25]. A detailed description of the model and the choice of parameter values is provided in Supplementary Note 1.

We use the model to determine the effect of environmental factors and cell

history (i.e. environments experienced in the past) on the relationship between cell density and CSP concentration. Crucially, the model assumes that all cells are homogeneous and that competence is only regulated by CSP, whose production increases with cell density since cells release all CSP they produce to a common extracellular pool. We are interested in determining whether these assumptions are sufficient to explain our experimental results in well-mixed cultures or if additional mechanisms need to be incorporated (e.g. density-independent competence induction and cell-cell contact dependent competence transmission [15,16,19]).

Competence develops at a critical CSP concentration

It has been reported that competence develops at a fixed time after inoculation from acid to alkaline conditions (pH 6.8 → 7.9) regardless of the inoculum size [15,19]. This observation has motivated the view that competence develops independently of cell density and rather acts as a timed response at the single cell level to the pH shift occurring at the moment of inoculation. We extended previous studies by exploring a wider range of inoculation densities (OD_{595nm}: 10^{-1} to 10^{-7}) (approximately 10^8 to 10^2 cells ml^{-1}) and preculturing conditions. We used the encapsulated serotype 2 strain *S. pneumoniae* D39 [26] and cells were washed before inoculation to remove CSP produced during the preculture. Importantly, we verified that CSP is actually present in the supernatant of competent cultures of strain D39 (Supplementary Fig. 1). To monitor competence development, the ComX-dependent promoter of the late competence gene *ssbB* was fused to the firefly *luc* gene and inserted at the non-essential *bgaA* locus. Activation and expression of *ssbB* is a good reporter for competence development since SsbB expression strongly correlates with actual transformation with externally

added DNA (e.g. refs. [13,27]).

As shown in Figure 2a, we find that the inoculation density in strain D39 does have an effect on the time of competence development, with competence initiating later for lower inoculum sizes. For instance, for the lowest inoculation density, competence initiates more than 4 hours later than for the highest inoculation densities (Figures 2a and left panel of 2c). Note that our luminometer can detect light from competent cells at an OD_{595} of $1.56x10^{-3}$ or higher (Supplementary Fig. 2) and therefore we cannot exclude the possibility that a very small subpopulation of cells initiates competence before we can detect it. Nevertheless, in all cases our estimates of the density of competence initiation are far higher than the detection threshold, indicating that competence in the majority of the population had not developed before crossing the density threshold (Figure 2c, right panel).

Importantly, we observe that the population density at competence initiation is not constant but positively related to the inoculation density. Hence, the dependency of the time of competence initiation on the inoculation density is not a consequence of competence developing at a fixed critical cell density for every condition. Instead, our results are consistent with the mathematical model, which predicts that competence develops when the CSP concentration has reached a critical threshold. The model shows that competence will start faster for higher inoculation densities because the CSP concentration reaches the critical threshold for competence activation earlier if more cells are producing CSP (Figure 2d, left panel). Moreover, the model shows that populations inoculated at low densities initiate competence at a lower density than populations inoculated at high densities consistent with the experimental data (right panels of Figures 2c and 2d). This is because cells inoculated at low cell densities already had time to start transcribing competence regulatory genes and accumulate

some CSP once they reached the same cell density of cultures freshly inoculated at a higher cell density (Figure 2e). Thus, the critical CSP threshold is reached sooner for low-density inoculated cultures. Notably, a common misconception in the field is that in a QS system the critical concentration of autoinducer should always be attained at the same fixed cell density [15,16,19,20].

It is well known that the pH of the medium affects competence development, with natural competence being inhibited under acid conditions [11,28]. Under our experimental conditions, competence only naturally develops in alkaline growth medium with a pH > 7.4. So far, we have studied competence with cells precultured in a non-permissive pH for competence development (pH 6.8). These preculture conditions were reflected in the model simulations by assuming that cells initially were in the competence-off state. We also simulated the alternative scenario that cells are already competent at inoculation. For this cell history, the model predicts that the time of competence initiation is lower, but only for high inoculation densities (Figure 2d, left panel). This happens because when cells are competent initially and are inoculated at high density they can produce enough CSP to remain competent (Supplementary Fig. 3). However, when inoculation density is low, cells cannot produce enough CSP and initial competence switches off. The timing of the subsequent competence initiation is then the same as if cells were not competent when inoculated.

To verify the predicted effect of cell-history on the timing of competence initiation, we controlled the competence state of cells at inoculation by manipulating the pH during preculture. Specifically, we compared the time of competence initiation for cells coming from a non-permissive (pH 6.8) and a permissive (pH 7.9) pH history for competence development. For inoculation densities below OD_{595nm} of 0.01, the pH of the preculture did not have an effect on the timing of competence initiation as

predicted by the model (Figures 2b and c). On the other hand, for inoculation densities above OD_{595nm} 0.01, there was a time delay in competence initiation for cells with an acid history whereas cells with a non-acid history were competent when inoculated and remained competent afterwards. This suggests that when the inoculation density is high, there are enough cells to take the CSP concentration above the threshold for competence activation if they are already producing CSP – as is the case of cells coming from a non-acid history. By contrast, if cells come from a non-permissive pH for competence development, the machinery for CSP production needs to be activated. This causes a delay in competence initiation at high cell densities, which, at least for our strain, would not result from regulation by a cell-density independent timing device[15,16,19].

To further study competence in conditions closer to what *S. pneumoniae* experiences in nature, we monitored competence development at the single-cell level in microcolonies growing on a semi-solid surface. To this end, *ssbB* was fused to *gfp* and competence initiation was followed using automated fluorescence time-lapse microscopy. We observed that competence synchronizes in neighboring microcolonies that are not in direct physical contact with each other (Figures 3A, 3B and Supplementary Movie 1). In addition, more than 95% of the population became competent in sharp contrast with competence development in other species such as *Bacillus subtilis* where less than 20% of the population enters the competent state [29,30]. Both observations are consistent with the view that CSP diffuses extracellularly and drives competence development across the population. We provide experimental evidence for this claim by studying mixed populations of our wildtype D39 strain and a *comC*$^{-}$ mutant, which is unable to produce and export CSP, and therefore only develops competence in the presence of external CSP. We followed competence

development in these mixed populations and found that competence can propagate from the wildtype to the *comC*⁻ mutant without the need of cell-to-cell contact (Figure 3C and Supplementary Movie 2). Note that the *comC*- mutant does not activate competence when grown alone (Supplementary Fig. 4 and Supplementary Movies 3 and 4). This implies that CSP can diffuse extracellularly which confirms our previous finding in liquid culture that CSP can be recovered from the supernatant of a competent culture (Supplementary Fig. 1). It is worth noting that although cells release CSP to an extracellular pool, a fraction might remain attached to them or in close proximity due to diffusivity on the polyacrylamide surface. This can introduce variation in the microenvironment that different cells experience and therefore in the extent of synchronization of competence initiation.

In the experiments discussed so far, we used an encapsulated strain, in contrast to the studies of Claverys *et al*. [15] and Prudhomme *et al*.[19], which were mainly based on unencapsulated strains. We explored whether this could explain our different observations regarding the effect of inoculation density on competence development by studying additional strains: an unencapsulated version of our strain D39, the clinical isolate PMEN14 together with its unencapsulated version and *S*. *mitis*, which is naturally unencapsulated. Although we still observe that competence develops later for smaller inoculums, the slope of the RLU signal decreases with inoculation density for the two capsule knockouts and *S*. *mitis*, in agreement with the results of Claverys *et al*.[15] and Prudhomme *et al*.[19] (Supplementary Fig. 5). A decreasing slope of the RLU signal likely indicates decreased synchronization in competence development at lower inoculation density. Since cells are synchronized by the common extracellular CSP pool, a possible scenario is that the absence of a capsule impedes cells from exporting all the CSP they produce. This would translate into less synchronization especially at

low inoculation densities. In the extreme scenario where cells would not share any CSP with other cells, competence regulation would be in fact independent from cell density. Note, however, that for the capsule knockout of our strain we can still detect CSP in the cell-free supernatant and also observe competence propagation in the absence of cell-cell contact (Supplementary Fig. 6 and Supplementary Movie 5).

pH and competence development

In order to understand how environmental factors affect competence, we quantified the effect of external pH on natural competence development. We studied competence at a fine-grained range of pH values from 6.8 to 8.5 and found a clear-cut value that separated permissive from non-permissive external pH values for natural competence development as reported before [11,27]. For our media this was pH 7.4 (Figure 4a). However, not only competence always developed at pH higher than 7.4 but the critical cell density for competence initiation decreased with increasing pH (Figure 4a and b). Therefore, pH does not relate to competence as a binary permissive/non-permissive condition but competence development is more efficient in more alkaline media. The data suggests that for non-permissive pH conditions the cell density at which competence would initiate is above the carrying capacity of the medium, which was also previously proposed by Chen and Morrison[11].

To study the effect of pH on competence we deleted *comC* from the *comCDE* operon and put it under the control of an IPTG-inducible promoter [31] at an ectopic locus. We then tested competence development in this strain at different pH values (7.2, 7.4, 7.6) and at varying IPTG concentrations. For any given IPTG concentration competence always developed earlier at higher pH. This indicates that cells need to express more *comC* to reach the critical CSP threshold for competence activation the

lower the pH (Supplementary Fig. 8). In fact, at low IPTG concentrations, competence only develops above pH 7.4. The same pattern is observed in an IPTG-inducible *comCDE* strain: for a fixed level of *comCDE* expression, the time of competence initiation still decreases with the pH (Supplementary Fig. 8). Remarkably, in this genetic background competence hardly develops at pH 7.2 even when *comCDE* is fully induced. Cells might need to express more *comC* at lower pH because CSP export and/or detection reduces with decreasing pH. This would also explain why competence barely develops at low pH in the IPTG-inducible *comCDE* strain since for a fixed level of *comE* expression, reduced CSP export and/or detection would bias the ComE~P/ComE ratio towards no competence development. We tested whether pH affects CSP detection by using a *comC*$^{-}$ mutant. We performed experiments with medium at different initial pH values where we added various concentrations of synthetic CSP, using the *comC*$^{-}$ mutant. We found that competence was mainly dependent on the CSP concentration and only minor differences were found among media with different pH (Supplementary Fig. 8). This suggests that competence development is not mainly mediated by pH-dependent CSP detection so possibly it is mediated by pH-dependent export. As peptidase-containing ATP-binding cassette transporters such as ComAB require ATP to transport substrates [32], it might be that the proton motive force influences its activity. Therefore, we incorporated the effect of pH in our model by changing the rate at which cells export CSP. In agreement with the experimental results, the modified model confirms that the density of competence initiation decreases with the rate of export of CSP and thus with higher pH. Also, the model predicts that for rates of CSP export below a certain threshold competence does not develop any more since cells never manage to accumulate enough CSP for competence to initiate (Figure 4c). Note however that this is a simplification of the

295 effect of pH in competence regulation since pH might also affect ComD and/or the
296 stability of CSP (as in other QS systems [6]) and as our data suggest it might also be
297 involved in the shutdown of competence (Supplementary Fig. 8). However, regardless
298 of the exact mechanism, as long as higher pH increases the rate at which single cells
299 produce and/or sense CSP, our model predicts that the density at which the critical
300 CSP concentration for competence activation is attained will decrease with increasing
301 pH (see Section C of the Supplementary Note 1 for a simple mathematical argument).
302 Finally, we assessed the joint effect of pH and cell density on competence
303 regulation. We did this by studying competence initiation for cultures inoculated at
304 different cell densities in media with different pH both experimentally and using the
305 model. The model predicts that competence will initiate earlier both for higher
306 inoculation densities and more alkaline pH (Figure 5a left panel): While higher
307 inoculation densities mean that more cells will start producing CSP after inoculation,
308 higher pH increases the rate at which individual cells produce CSP. The experimental
309 data is consistent with this prediction (Figure 5b left panel). Therefore, the observation
310 that pH affects competence development is not conflicting with regulation by cell
311 density because the CSP concentration depends on both of these factors.
312

313 **Induction of competence by antibiotics**

314 The induction of pneumococcal competence is affected by the presence of certain
315 classes of antibiotics [13,27], which has been considered additional evidence for the
316 hypothesis that competence can be regulated independently of cell density [13,15]. We
317 evaluated this claim by studying the role of HPUra and streptomycin on competence
318 regulation. We chose these antibiotics since the mechanisms by which they induce
319 competence at the molecular level have been elucidated to some extent: HPUra stalls

replication forks during DNA replication while initiation of DNA replication continues, thereby increasing the copy number of genes near the origin of replication (*oriC*). As a consequence, it up-regulates transcription of *comAB, comCDE* and *comX* as these operons are located proximal to *oriC* [27]. Streptomycin causes mistranslation and is thought to regulate competence via the membrane protease HtrA which targets misfolded proteins and also represses competence possibly by degrading CSP [33] (also see Supplementary Fig. 9). By increasing the amount of misfolded proteins, streptomycin could reduce the rate at which CSP is degraded by HtrA leading to competence induction.

We reproduced the effect of HPUra and streptomycin on competence regulation in our model by increasing the transcription rate of *comAB, comCDE* and *comX* and by reducing the rate at which CSP degrades, respectively. Our model predicts that the presence of antibiotics lowers the pH threshold for competence development (Figure 5a) since antibiotics can counteract the effect of acidic pH to the point that cells can still accumulate enough CSP to become competent. They do this by increasing the rate at which single cells produce CSP (reducing the number of cells needed to reach the critical CSP concentration for competence initiation) or by increasing the rate at which they sense CSP (reducing the critical CSP concentration for competence initiation). Also, it predicts that for pH values where competence is already induced without antibiotics, it will develop faster in the presence of antibiotics (Figure 5a). We tested these predictions experimentally using antibiotics at concentrations that have a minimal effect on growth since we did not incorporate growth reduction due to antibiotic stress in the model. In agreement with previous studies [13,27,33] and with the model predictions, we find that antibiotics can induce competence at pH values that are repressive for natural competence development (Figure 5b and c). We also find support

for the second prediction of the model since for permissive pH values for natural competence development (above 7.4), competence is induced earlier in the presence of antibiotics (Figure 5b and d). Remarkably, both the model and the experiments show that the combined effect of pH and cell density in the presence of antibiotics remains the same as when no antibiotics are added (compare left panel with middle and right panels in Figures 5a and 5b): Competence induction still occurs earlier for high densities of inoculation and more alkaline pH values. In the case of HPUra at pH 7.3 it is even possible to see that competence does not develop for the highest inoculation density as the population probably reaches carrying capacity before enough CSP is produced. Finally, note that there might be alternative mechanisms from the ones incorporated in the model by which antibiotic stress affects competence. For instance, antibiotics reduce growth and can induce stress responses that lead to global changes on transcription and translation.

Bistable region for competence development and cell history

An important feature of the competence regulatory network is the presence of a positive feedback that couples CSP detection to CSP production (Figure 1). Signaling systems that contain positive feedback loops often exhibit switch-like responses resulting in the occurrence of alternative stable states [34]. We varied the strength of the positive feedback loop in the model by changing the rate of CSP export and found that the competence regulatory network exhibits bistability for a range of intermediate CSP export rates. In this range, the model predicts the existence of two alternative states where competence switches 'ON' or 'OFF' depending on the initial conditions (Figure 6a).

Since in the model the rate of CSP export is positively correlated to the pH, we expected to find a region of pH values exhibiting similar bistability as an additional experimental corroboration of the model. Indeed, we found support for the existence of a bistable region at pH 7.4 where the wild type developed competence if CSP was externally added in concentrations above 4 ng mL^{-1} (Figure 6b). Thus, whereas competence always switched on for pH values above 7.4 regardless of the initial CSP concentration, for pH 7.4 both 'ON' and 'OFF' states were observed depending on the initial CSP concentration. Moreover, at pH 7.4 competence developed with 4 ng mL^{-1} of CSP a concentration that would not induce competence in the *comC*$^{-}$ mutant (Supplementary Fig. 8), which indicates that CSP production in the wild type was kick-started by the initial addition of CSP resulting in enough overall CSP for competence induction.

Bistable systems usually exhibit hysteresis. For this reason, we expected that at pH 7.4 where both the 'ON' and 'OFF' states are attainable, cell history, would influence competence induction. From our previous experiments we determined that cells coming from acid preculture inoculated at pH 7.4 do not develop competence at any density of inoculation (Figure 5b left panel, second column). We then studied whether there is history-dependence by inoculating cells coming from non-acid preculture at pH 7.4. We found that cells coming from a non-acid preculture became competent when inoculated at densities above OD_{595nm} of $2.4x10^{-4}$, which demonstrates that cell history can influence competence development at this pH (Figure 6c). Past history has an effect on competence because it determines the state of the machinery for CSP production, which is 'OFF' when cells come from acid preculture but 'ON' when they come from non-acid conditions. This explains why the effect of non-acid cell history appears from a minimum inoculation density, since enough cells need to be inoculated

in order for them to produce the amount of CSP necessary for the system to remain 'ON' (Figure 6a inset). We then hypothesized that at pH 7.3 the critical inoculation density of cells coming from non-acid history would have to be even higher than the one at pH 7.4 as the model predicted that a higher initial concentration of CSP would be necessary for the system to remain 'ON' at lower pH. We confirmed this prediction experimentally by showing that at pH 7.3 competence does not develop for an inoculation density of OD_{595nm} 2.4 x 10^{-4} (as for pH 7.4) but from 7.4 x 10^{-4} upwards (Supplementary Fig. 10). Thus, our results show that, as a consequence of the positive feedback involved in CSP production, previous exposure to different environmental conditions can determine whether competence is induced or not by modifying the state of the machinery for CSP production and/or sensing.

Discussion

Recently, the view that bacteria use autoinducers as QS signals has been debated since autoinducer concentration can change in response to the environment. Here, we show experimentally that cell density, pH and antibiotic stress simultaneously regulate competence development in *S. pneumoniae* (Figures 2-5), a system classically framed in the paradigm of QS. Using a mathematical model, we show that this occurs because pH and antibiotics modify the rates at which single cells produce and sense CSP and therefore the strength of the positive feedback loop coupling CSP detection to CSP production (Figures 4 and 5). This environmental dependency does not override regulation by cell density but rather modulates the relationship between the number of cells and the CSP concentration. A fundamental aspect to the dependency on cell density is that cells share CSP with others. Importantly, here we provide evidence both in liquid culture (Supplementary Fig. 1) and through single-cell observations (Figure

418 3) that CSP is exported to the extracellular space. Finally, we show that competence
419 development is history-dependent since past environmental conditions can modify the
420 status of the machinery to produce and respond to CSP determining whether
421 competence switches on or not (Figure 6). Hysteresis in the competence response
422 might be especially important in the natural niche of the pneumococcus, the human
423 nasopharynx. In particular it is consistent with the observation that there is constitutive
424 upregulation of competence in pneumococcal biofilms during nasopharyngeal
425 colonization [35]. In this context, once competence is triggered for the first time, cells
426 would be primed to rapidly initiate another round of competence.

427 Why is competence controlled by CSP? CSP does not act as a timing device in our
428 encapsulated strain since competence develops in a cell-density dependent manner
429 without the necessity of cell-cell contact [15,16,19] (Figures 2 and 3). Regarding the
430 hypothesis that CSP is a probe to test diffusion [20], our results suggest that focusing on
431 diffusion alone oversimplifies the information and functionality that cells can gather
432 through CSP production. We hypothesize that by using an autoinducer peptide,
433 bacteria can coordinate the development of competence and in particular the
434 expression of fratricins and bacteriocins, which are under the control of the competent
435 state. These proteins can lyse or inhibit the growth of surrounding cells that are not
436 competent, increasing the efficiency of genetic transformation and mediating
437 competition with other bacteria [36–39]. By coordinating competence expression via CSP,
438 an isogenic bacterial population can increase the total concentration of secreted
439 fratricins and bacteriocins in times where population density is high, which likely
440 translates into a higher amount of lysed cells and therefore potential DNA donors.
441 Importantly, coordinating competence expression can also prevent the killing of clonal
442 siblings since immunity to these proteins comes with the competent state. Note

443 however that the extent to which cells synchronize competence development may vary
444 depending on the strain, genotype and growth conditions. In particular, our results
445 suggest that unencapsulated strains may synchronize less, which would explain the
446 difference between the findings reported by Prudhomme et al.[19] and our study.
447 Decreased synchronization may result from cells exporting less CSP to the
448 extracellular space and keeping more to themselves. In fact, in other species like *S.*
449 *thermophilus* where competence is controlled by ComS, a peptide that rapidly gets
450 imported back into the cell, the rate of competence development decreases with the
451 inoculation density [40] as observed for the unencapsulated pneumococcal strains.
452 Finally, note that *S. pneumoniae* grows primarily in biofilms where there is
453 heterogeneity in the physiological status and microenvironment that different cells
454 experience. This can certainly influence the degree of synchronization in competence
455 initiation across a population especially in the light of our findings that both current
456 and past environmental conditions affect the competence regulatory network. Indeed,
457 recent work in *S. mutans* showed heterogeneous competence activation of cells within
458 biofilms and upon different environmental pH ranges [41,42]. While studies of well-mixed
459 cultures give insight into the response mechanism shared by all cells in a population,
460 additional work is needed in the future to study how CSP production and detection by
461 individual cells is shaped by their spatial context and history and to unravel how
462 individual responses translate into patterns of population synchronization across
463 different genotypes and strains.

464 What is the relevance of the information carried by CSP? Alkaline pH and
465 antibiotic stress can induce competence by increasing the rate at which single cells
466 produce and sense CSP. We expect this to be a general mechanism by which sources
467 of stress that are alleviated through competence induce this state (e.g. mobile genetic

468 elements as hypothesized by Croucher et al.[43]). Upregulating competence in the
469 presence of antibiotics can increase survival by activating the expression of stress
470 response genes [7,18], facilitating repair of damaged DNA and mediating acquisition of
471 resistance [18,44]. Our findings suggest that strategies to prevent competence
472 development in response to antibiotics can focus on counteracting the effect of
473 antibiotics on the rate at which cells produce or sense CSP. Regarding the benefits of
474 upregulating competence with alkaline pH, these are less clear and could be an
475 example of a non-adaptive response resulting from the inherent biochemical properties
476 of ComAB and possibly ComD. Importantly, CSP can integrate additional
477 environmental cues like oxygen availability through the CiaRH two component
478 system, which represses *comC* post-transcriptionally and is required for virulence
479 expression and host colonization[14,45]. In fact, CiaRH is key for the regulation of
480 competence by multiple environmental signals in other streptococci like *S. mutans* [46].

481 Our findings support the view that functional hypotheses stressing individual
482 factors like diffusion or population density underplay the complexity of information
483 integrated by QS systems [4,47–51]. Although the term 'quorum sensing' overemphasizes
484 the role of population density, we advocate for keeping it due to its widespread use and
485 the fact that density will modify autoinducer concentration in any autoinducer
486 production system. Crucially, QS should be used in a broad sense that acknowledges
487 that bacteria integrate past and current environmental factors in addition to population
488 density into their QS responses. This view might be very useful for other autoinducer
489 production systems like competence in *Vibrio cholerae*, where the synthesis of the
490 autoinducer, CAI-1, depends on the intracellular levels of cAMP-CRP and therefore
491 might incorporate information on the metabolic status of the cell [52,53]. Also in other
492 systems, clear links between signal production, quorum threshold and environmental

493 conditions have been shown to affect QS [54–58].

494 Given that many biotic and abiotic factors can modify autoinducer concentrations
495 [59], future work should aim to study the relevance of such factors in the natural context
496 where bacteria secrete autoinducers. Such work is crucial to assess whether
497 upregulating QS in response to a particular factor provides a benefit for bacteria or is
498 merely a result of the biochemical properties of the QS regulatory network. An
499 interesting possibility is that, as in other biological systems [60], bacteria could perform
500 collective sensing of the environment through social interactions. In this context, by
501 secreting autoinducers cells could share individual estimates of environmental
502 conditions (e.g. antibiotic stress) for which upregulating QS is beneficial. Then,
503 autoinducer secretion would provide a way to get a more reliable estimate of the
504 environmental conditions by allowing a population to pool estimates made by
505 individual cells. Importantly, such a role for autoinducer secretion would explain the
506 dependency of QS on both cell density and the environment.

507

508 **Methods**

509 **Bacterial strains and growth conditions**

510 All pneumococcal strains used in this study are derivatives of the clinical isolate *S.*
511 *pneumoniae* D39 [26] unless specified otherwise. To monitor competence development,
512 strains either contain a transcriptional fusion of the firefly *luc* and the *gfp* gene with the
513 late competence gene *ssbB* or a full translational *ssbB-gfp* fusion. Cells were grown in
514 in C+Y complex medium at 37ºC. C+Y was adapted from Adams and Roe [61] and
515 contained the following components: adenosine (65 µm), uridine (107 µM), L-

asparagine (331 µM), L-cysteine (71 µM), L-glutamine (150 µM), L-tryptophane (29.4) µM, casein hydrolysate (5 g L^{-1}), BSA (8 mg L^{-1}), biotin (2.46 µM), nicotinic acid (4.87 µM), pyridoxine (3.4 µM), calcium pantothenate (5.04 µM), thiamin (1.9 µM), riboflavin (0.744 µM), choline (48 µM), $CaCl_2$ (113 µM), K_2HPO_4 (48.8 mM), $MgCl_2$ (2.46 mM), $FeSO_4$ (1.8 µM), $CuSO_4$ (2 µM), $ZnSO_4$ (1.74 µM), $MnCl_2$ (40 µM), glucose (11.1 mM), sodium pyruvate (2.7 mM), saccharose (944 µM), sodium acetate (24.4 mM) and yeast extract (2.5 g L^{-1}).

Construction of recombinant strains

To transform *S. pneumoniae*, cells were grown in C+Y medium (pH 6.8) at 37ºC to an OD_{595} of 0.1. Then, cells were treated for 12 minutes at 37ºC with synthetic CSP-1 (100 ng mL^{-1}) and incubated for 20 minutes at 30ºC with the transforming DNA. After incubation with the transforming DNA, cells were grown in C+Y medium (pH 6.8) at 37ºC for 90 minutes. *S. pneumoniae* transformants were selected by plating inside Columbia agar supplemented with 3% of defibrinated sheep blood (Johnny Rottier, Kloosterzande, The Netherlands) and the appropriate antibiotics. Working stocks of cells were prepared by growing cells in C+Y (pH 6.8) until an OD_{595} of 0.4. Cells were collected by centrifugation (1595 x *g* for 10 minutes) and resuspended in fresh C+Y medium (pH 6.8) with 15% glycerol and stored at -80ºC. See the details on the construction of the strains below and see Supplementary Table 1 for a list of all the strains used in this study.

Strain DSM2

To follow competence development during pneumococcal growth, a transcriptional fusion of two reporter genes, *luc* (firefly luciferase) and *gfp*, to the late competence promoter P_{ssbB} was used. The plasmid pLA18 [27], containing the P_{ssbB}-*luc*-*gfp* construct

was transformed into *S. pneumoniae* D-PEP1 [62] and transformants were selected on Columbia blood agar supplemented with 1 µg mL^{-1} tetracycline. The P_{ssbB}-*luc*-*gfp* construct integrates into the *bgaA* locus, and correct transformants were verified by PCR.

Strains ADP243 and ADP62

To prevent the production of CSP, the original *comC* gene was replaced by an erythromycin resistance marker, leaving the promoter and therefore the polycistronic nature of *comDE* intact. The upstream region was amplified using primers LAG11 (GGCGGATCCGGCAGTTTGTGTAATAGTAC) and ADP2/48+AscI (ACGTGGCGCGCCGTTCCAATTTAACTGTGTTTTTCAT), the downstream region with primers ADP2/47+BamHI (ACGTGGATCCGAAATAAGGGGAAAGAGTAATGGATTTATTTG) and LAG54 (AATCGCCATCTTCCAATCCC), and the erythromycin resistance marker with 0292_ery_R+BamHI (GCGGATCCTGTCTTTGACCCAATCATTC) and sPG19+AscI (ACGTGGCGCGCCCGGAGGAATTTTCATATGAAC). All three fragments were digested with the proper restriction enzymes (AscI and/or BamHI) and ligated. The Δ*comC::ery* fragment containing the erythromycin resistance marker flanked by the sequence up- and downstream of *comC* without altering the natural transcription of *comDE*- was transformed into D39 and DLA3[27] resulting in strains ADP243 (Δ*comC::ery*) and ADP62 (Δ*bgaA*:: P_{ssbB}-*luc*, Δ*comC::ery*), respectively. Transformants were selected on Columbia blood agar containing 0.25 µg mL^{-1} erythromycin. Correct deletion of *comC* was verified by PCR and sequencing.

Strains ADP49 and ADP51

To monitor natural competence in two clinical isolates, the PCR product of the

564 fragment *ssbB-luc-kan* from strain MK134[27] was transformed into the pneumococcal
565 strain PMEN14 and the *Streptococcus mitis* strain NTCC10712. Transformants were
566 selected on Columbia blood agar containing 250 µg mL^{-1} kanamycin resulting in
567 strains ADP49 (PMEN14, *ssbB-luc*) and ADP51 (*S. mitis ssbB-luc*), respectively.

568 ***Unencapsulated strains ADP25, ADP26, ADP92***

569 To delete the polysaccharide capsule, the whole *cps* operon was replaced with a
570 chloramphenicol resistance cassette. The primers used were: ADP1/51
571 (CGGTCTTCAGTATCAGGAAGGTCAG) and ADP1/52+AscI
572 (CGATGGCGCGCCCTTCTTTCTCCTTAATAGTGG) to amplify the upstream
573 region; primers ADP1/53+NotI
574 (CACGGCGGCCGCGAGAAAGTTTTAAAGGAGAAAATG) and ADP1/54
575 (GATAGAGACGAGCTGCTGTAAGGC) to amplify the downstream region;
576 sPG11+AscI (ACGTGGCGCGCCAGGAGGCATATCAAATGAAC) and
577 sPG12+NotI (ACGTGCGGCCGCTTATAAAAGCCAGTCATTAG) to amplify the
578 chloramphenicol cassette. Products were digested with the appropriate enzymes (AscI
579 and/or NotI) and ligated. Strains D39, DLA3 and ADP49 were transformed with the
580 ligation product and transformants were selected on Columbia blood agar containing
581 4.5 µg mL^{-1} chloramphenicol resulting in strains ADP25 (Δ*cps::chl*), ADP26 (Δ*bgaA*::
582 P_{ssbB}-*luc*, Δ*cps::chl*), ADP92 (PMEN14, *ssbB-luc*, Δ*cps::chl*).

583 ***Strains ADP249 and ADP151***

584 To follow competence development of micro-colonies during time-lapse microscopy,
585 the PCR product of the C-terminal fusion of *ssbB* with *gfp* and a downstream
586 kanamycin cassette were amplified using chromosomal DNA of strain RA42 as a
587 template [63] and transformed into the D39 strain and into its unencapsulated variant

ADP25 resulting in strains ADP249 (P_{ssbB}-*ssbB-gfp*) and ADP151 (P_{ssbB}-*ssbB-gfp*, Δ*cps::chl*), respectively. Transformants were selected on Columbia blood agar containing 250 µg mL^{-1} kanamycin.

Strains ADP247** and **ADP248

Plasmid pPEP43 [62] containing a constitutive promoter driving the expression of the red fluorescent protein mKate2 (cytoplasmic localization) was transformed into strain D39 and its unencapsulated derivative ADP25. pPEP43 integrates into the *cep*-locus, and transformants were selected on Columbia blood agar containing 125 µg mL^{-1} spectinomycin. The resulting strains, ADP235 (Δ*cep::p3-mkate2*) and ADP244 (Δ*cep::p3-mkate2*, Δ*cps::chl*), were transformed with the PCR product P_{ssbB}-*ssbB-gfp* including the kanamycin cassette (same as for strain ADP249). Transformants were selected on Columbia blood agar containing 250 µg mL^{-1} kanamycin. Finally, the resulting strains, ADP245 (Δ*cep::p3-mkate2*, P_{ssbB}-*ssbB-gfp*) and its unencapsulated variant ADP246 (Δ*cep::p3-mkate2*, Δ*cps::chl*, P_{ssbB}-*ssbB-gfp*), were transformed with the *comC::ery* fragment described above. This resulted in strains ADP247 (Δ*cep::p3-mkate2*, P_{ssbB}-*ssbB-gfp*, Δ*comC::ery*) and ADP248 (Δ*cep::p3-mkate2*, Δ*cps::chl*, P_{ssbB}-*ssbB-gfp*, Δ*comC::ery*). Transformants were selected on Columbia blood agar containing 0.25 µg mL^{-1} erythromycin.

Strain ADP95

To use an IPTG-inducible system, we first transformed the codon optimized *lacI* gene [31] into strain DLA3 [27]. For that, we PCR-ed the fragment including *lacI* integrated into the *prsA*-locus together with a gentamycin resistance cassette from chromosomal DNA of strain DCI23 [31] using primers OLI40 (CCATGGCATCAGCGAGAAGGTGATAC)

611 and OLI41 (GCGGCCGCAGGATAGAAAGGCGAGAG) and transformed the
612 resulting PCR product to strain DLA3 while selecting for gentamycin (20 μg mL^{-1})
613 resulting in strain ADP95 (D39, Δ*bgaA*::P_{ssbB}-*luc*, Δ *prsA*::*lacI*).

614 ***Strains ADP112 and ADP107***

615 To control the production of *comC*, and thereby CSP, a strain was constructed with an
616 ectopic copy of *comC* (at the *cep* locus) under control of the IPTG-inducible promoter
617 P_{lac} [31], and the subsequent deletion of the original *comC* (replaced by an erythromycin
618 resistance cassette). The inducible system was created using BglFusion cloning [62]. To
619 amplify the *comC* fragment, primers ADP2/38
620 (CAGTGGATCCGGTTTTTGTAAGTTAGCTTACAAG) and ADP3/31
621 (CAGTCTCGAGCCCAAATCCAAATAAATCCATTAC) were used using D39
622 chromosomal DNA as a template. To control the production of *comCDE*, a strain with
623 an IPTG-inducible *comCDE* was created, with the deletion of the original *comCDE*
624 locus. The inducible system was created as explained above. To amplify the *comCDE*
625 fragment, primers ADP2/38
626 (CAGTGGATCCGGTTTTTGTAAGTTAGCTTACAAG) and ADP2/58
627 (ACGTCTCGAGGCGGCCGCCAATTTCTTGCTAATTGTC) were used using
628 chromosomal DNA of D39 as a template. PCR products were digested with BglII and
629 XhoI and were ligated with similarly digested pPEP1 plasmid containing the promoter
630 P_{lac} [31]. The ligations were transformed into strain ADP95 and transformants were
631 selected on Columbia blood agar containing 125 μg mL^{-1} spectinomycin. To these
632 strains, either the PCR product Δ*comC::ery* (see above) or a PCR product containing
633 Δ*comCDE::chl* from strain MK135 [27] was transformed and transformants were
634 selected on Columbia blood agar containing either 0.25 μg mL^{-1} erythromycin or 4.5

635 µg mL^{-1} chloramphenicol resulting in strains ADP112 (Δ*bgaA*::P_{ssbB}-*luc*, Δ*cep*::P_{lac}-
636 *comC*, Δ*comC::ery*) and ADP107 (Δ*bgaA*::P_{ssbB}-*luc*, Δ*cep*::P_{lac}-*comCDE*,
637 Δ*comCDE::chl*), respectively.

638 ***Strain MK356***

639 To construct MK356 (Δ*bgaA*:: P_{ssbB}-*luc-gfp*, Δ*htrA::ery*), *htrA* was replaced with an
640 erythromycin resistance gene. A region upstream of *htrA* was amplified from genomic
641 DNA of *S. pneumoniae* D39 using primers htrA-up-F (5'-
642 GAACCTGCGACCGTTCGCTTAGAAGG-3') and htrA-up-R+BamHI (5'-
643 GCGCGGATCCTCCATATGTTTGAATTACTG-3') and a region downstream of *htrA*
644 was amplified with primers htrA-Dwn-F-EcoRI (5'-
645 CGCGGAATTCGACATCTATGTAAAGAAAGC-3') and htrA-Dwn-R (5'-
646 GCTGTTGATAATTCTACTATATTCTTC-3'). An erythromycin resistance gene was
647 amplified from plasmid pORI28 [64] using primers EmR-F+BamHI (5'-
648 GCGCGGATCCTATGAACGAGAAAAATATAAAACAC-3') and EmR-R+EcoRI
649 (5'-CGCGGAATTCGCAGTTTATGCATCCCTTAACTTAC-3'). The three fragments
650 were digested with appropriate restriction enzymes (BamHI and/or EcoRI) and ligated.
651 The Δ*htrA::ery* fragment, containing the an erythromycin resistance gene flanked by
652 the sequence up- and downstream of *htrA*, was transformed into strain DSM2.
653 Transformants were selected on Columbia blood agar containing 0.25 µg mL^{-1}
654 erythromycin. Correct deletion of *htrA* was verified by PCR.

655 **Density and luminescence assays**

656 Cells were pre-cultured in acid C+Y (pH 6.8) or in non-acid C+Y (pH 7.9) at 37ºC to
657 an OD_{595nm} of 0.1. Right before inoculation, they were collected by centrifugation
658 (8000 rpm for 3 minutes) and resuspended in fresh C+Y (pH 7.9). Unless indicated

otherwise all experiments were started with an inoculation density of OD_{595} 0.002 with cells from an acid preculture. Luciferase assays were performed in 96-wells plates with a Tecan Infinite 200 PRO luminometer at 37ºC as described before [62]. Luciferin was added at a concentration of 0.5 mg mL^{-1} to monitor competence by means of luciferase activity. Optical density (OD_{595nm}) and luminescence (relative luminescence units [RLU]) were measured every 10 minutes. The time and density of competence initiation correspond to the first time point where the RLU signal is equal or above 200 units. RLU is used instead of RLU/OD for Figure 2 because 1) when competence develops the rate at which the RLU signal increases is faster than the growth rate and 2) due to the very low inoculation densities used for Figure 2 the RLU/OD can be very high at the start (clearly before competence has developed). The value of 200 units was chosen because once this value is reached competence always developed. The effect of pH on competence development was studied by inoculating cells in C+Y at a range of pH values from 6.8 to 8.5. pH was adjusted by adding HCl and NaOH. The effect of antibiotics was studied by adding streptomycin (3 µg mL^{-1}) and HPUra (0.075 µg mL^{-1}) to C+Y.

Detection of CSP in cell free supernatant

S. pneumoniae D39 wild-type and its *comC* deficient version, ADP243, were grown in non-acid C+Y (pH 7.9) at 37ºC to an OD_{595nm} of 0.1. Cells were spun down by centrifugation at 20000 x g for 5 min. The supernatant was sterilized by filtering twice through 0.2 µm filters. The supernatant was plated on Columbia blood agar to confirm that no viable cells were present. For both the wild-type and the *comC*-deficient strain, the cell-free supernatant was diluted 1:1 with 2x concentrated C+Y medium containing luciferin, and pH was adjusted to the indicated value by addition of HCl. The indicator

683 strain DSM2 pre-grown in C+Y pH 6.8 was then inoculated, and growth and luciferase
684 activity was monitored as described before.

685 **Time-lapse fluorescence microscopy**

686 A polyacrylamide slide was used as a semi-solid growth surface to spot the cells for
687 time-lapse microscopy. This slide was prepared with C+Y (pH 7.9) and 10%
688 acrylamide as reported previously [65]. Cells were pre-cultured in acid C+Y (pH 6.8) and
689 right before inoculation on the slide they were resuspended in fresh C+Y (pH 7.9) as
690 explained before. Phase contrast, GFP and RFP images were obtained using a
691 Deltavision Elite microscope (GE Healthcare, USA). Time-lapse videos were recorded
692 by taking images every 10 minutes after inoculation unless specified otherwise. File
693 conversions were done using Fiji and analysis of the resulting images was done using
694 Oufti [66].

695 **Mathematical model**

696 A mathematical model of the network of competence regulation (Figure 1) was
697 developed as a system of ODEs. The model incorporates the protein interactions
698 involved in sensing CSP via the two-component system formed by ComD and ComE
699 and exporting it via ComC and ComAB. Additionally, it explicitly models the
700 interaction of ComE and ComE~P with the gene promoters of *comAB*, *comCDE* and
701 *comX*. This is important since ComE~P binds these promoters as a dimer introducing
702 non-linearity into the system, which underlies the observed bistability. Population
703 growth is logistic and it is assumed that cells are homogeneous and that they export all
704 CSP they produce to a common extracellular pool. See Supplementary Note 1 for the
705 equations and further description.

706

Data availability

The authors declare that the data supporting the findings of the study are available in this article and its Supporting Information files, or from the corresponding authors upon request.

712

713 **Author Contributions**

714 SMG, AD, RAS, MK, GSvD, and JWV designed research; SMG, AD, RAS and MK
715 performed experiments; SMG and GSvD developed the model; SM, AD, RAS, MK,
716 FJW, GSvD, and JWV analyzed data; and SMG, FJW, GSvD and JWV wrote the
717 paper.

718

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728

729 **Conflicts of interest**

730 The authors state no conflicts of interest.

731

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919

Figure legends

Figure 1. Network of competence regulation in *S. pneumoniae*. ComC (C) binds the membrane protein complex ComAB, and it is processed and exported as CSP to the extracellular space. CSP binds to the histidine kinase ComD, which is located in the membrane as a dimer. Upon CSP binding, ComD autophosphorylates and transfers the phosphate group to the response regulator ComE [9,23]. The phosphorylated form of ComE (ComE~P) dimerizes and activates transcription of *comAB*, *comCDE* and *comX* by binding to their promoters [8,9]. Unphosphorylated ComE can also bind these promoters, repressing their transcription [23,67]. Synthesis of the alternative sigma factor ComX directs transcription of genes required for genetic transformation as well as other functions [7,24]. Two key features of this network are the presence of a positive feedback loop (since increasing CSP detection leads to increasing CSP production) and of non-linearity (since ComE~P interacts with the gene promoters as a dimer).

Figure 2. Competence is regulated by cell density. (**a**,**b**) Growth curves (OD_{595nm}) and competence expression measured as relative luminescence units (RLU) expressed from the promoter of the late competence gene *ssbB* for populations inoculated at a range of densities and grown in C+Y medium with initial pH 7.9. In **a**, cells were precultured in acid conditions (pH 6.8), while in **b** cells were precultured in non-acid conditions (pH 7.9). Four replicates are shown for each of seven inoculation densities (OD_{595nm}): 0.1 (Green), 0.05 (Red), 0.01 (Blue), 10^{-3} (Purple), 10^{-4} (Light green), 10^{-5} (Yellow) and 10^{-6} (Brown). Competence does not develop in cells coming from acid preculture and inoculated at a density of 0.1. (**c**) Effect of inoculation density on the

time until competence initiation (left panel) and the population density at which competence was initiated (right panel). Competence initiation was defined as the time where the RLU signal exceeded 200 units. Note that our luminometer has enough sensitivity to detect light from competent cells at a density of $1.56x10^{-3}$ or higher even if they correspond to a subpopulation (Supplementary Fig. 2). (**d**) Predictions of the mathematical model concerning the effect of inoculation density on the timing of competence initiation (left panel) and the density at which competence initiates (right panel). In the model, competence initiation was defined as the time where the total concentration of ComX times the population density exceeds 2000 units. Non-acid preculture is simulated in the model by setting the initial amount of all proteins in the competence regulatory network to the value they attain when cells are competent. (**e**) The model predicts that populations inoculated at lower densities will reach a threshold CSP concentration (dotted line) at a lower density than populations inoculated at higher densities.

Figure 3. Competence can propagate by CSP diffusion without the necessity of cell-to-cell contact. (**a**) Phase contrast and GFP images (left panel) of a set of neighboring microcolonies of D39 with a fusion of the late competence gene *ssbB* to *gfp* (P_{ssbB}-*ssbB*-*gfp*). The first images (t = 0 min) were taken right after inoculation on the microscopy slide and the second ones correspond to the moment of competence initiation (t = 65 min). Images were taken every 5 minutes. Crucially, when competence starts the microcolonies are not in direct physical contact with each other. Analysis of the fluorescence signal across the entire population (right panel) shows that once competence starts, after 60 minutes from inoculation, the distribution of fluorescence intensity moves to higher intensity values through time. We set as a

969 threshold for counting competent cells a fluorescence signal 50% above background.
970 After 85 minutes, we counted 97 out of 99 cells as competent cells. (b) Distribution of
971 fluorescence signal within each microcolony in the window of competence initiation.
972 The microcolony numbers correspond to the ones indicated in the phase contrast image
973 in panel a). Competence initiates in all microcolonies within a window of 5 minutes.
974 (**c**) Time-lapse fluorescence microscopy tracking competence development in two
975 colonies with a fusion of the late competence gene *ssbB* to *gfp*: one formed by cells of
976 D39 (ADP249) and one formed by cells of a *comC* deficient D39 (ADP247). The two
977 strains are distinguishable because ADP247 constitutively expresses a red fluorescent
978 protein. Competence developed first in D39 and then it propagated to the *comC*$^-$
979 mutant after 20 minutes without the necessity of cell-cell contact. Note that we
980 checked that the *comC* deficient D39 (ADP247) does not become competent by itself
981 when grown without D39 (Supplementary Fig. 4 and Supplementary Movies 3 and 4).
982 The scale bar is 5 μm in all images.

983

984 **Figure 4**. **Competence is upregulated by higher pH.** (**a**) Effect of initial medium pH
985 on growth curves (left panel) and the dynamics of competence expression (middle
986 panel). Competence expression was quantified as relative luminescence units (RLU)
987 normalized by cell density. In the right panel, competence expression is plotted in
988 relation to cell density. All populations were grown at the indicated initial pH and
989 inoculated at a density of OD_{595nm} 0.002. Three replicates are shown for each initial
990 pH. **b**) Effect of initial medium pH on the population density at which competence
991 was initiated (the density at which RLU exceeded 200 units). Competence did not
992 develop at pH 7.4 and below. Note that the indicated pH is the initial pH of the

993 medium, which does not stay constant due to acidification by growth (Supplementary
994 Fig. 7). Although the pH drops considerably in fully-grown cultures, acidification is
995 still minor at the density where competence develops. **c**) Predictions of the model on
996 the effect of the rate of CSP export, r_e, and thus the pH, on the density of competence
997 initiation. Competence does not develop anymore below a threshold rate of CSP
998 export.

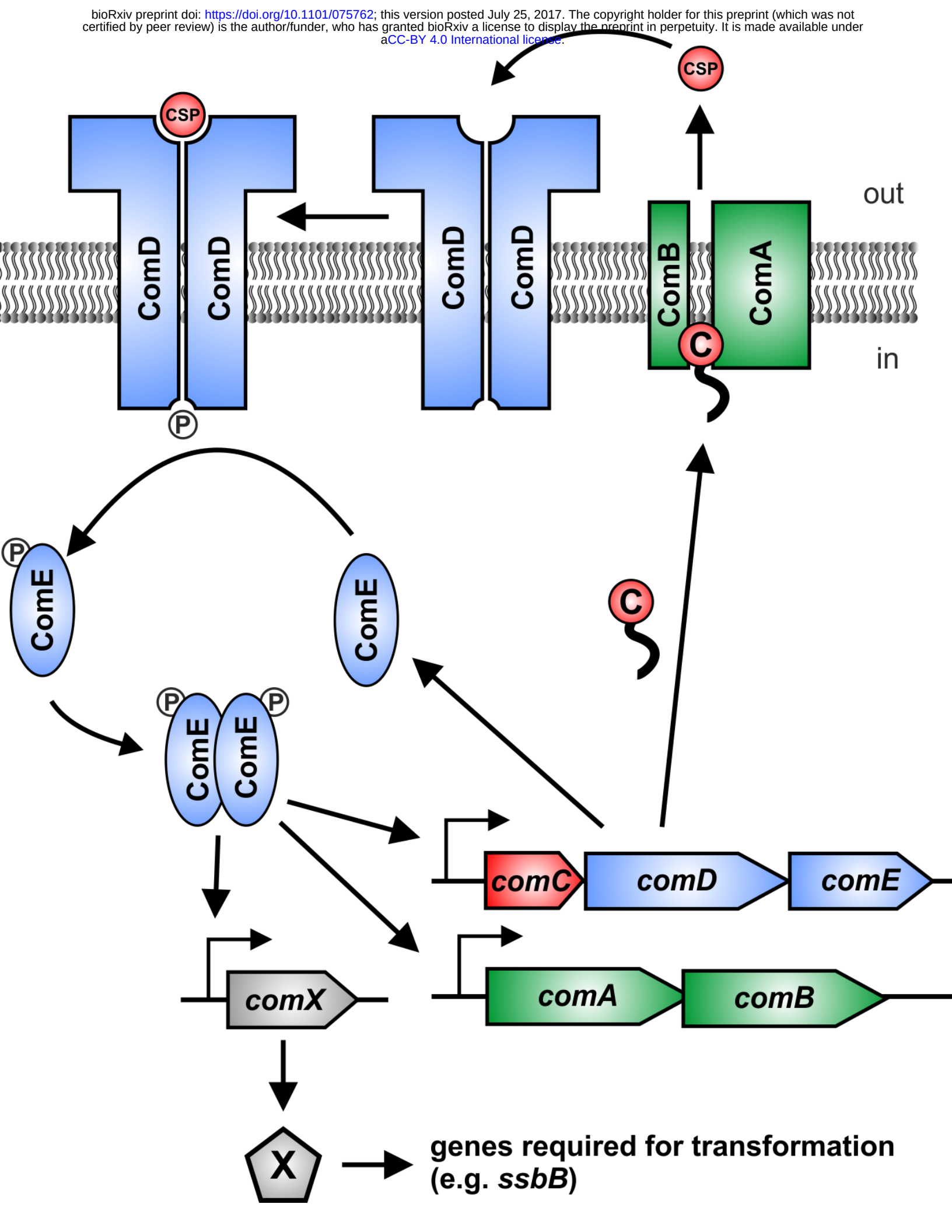
999

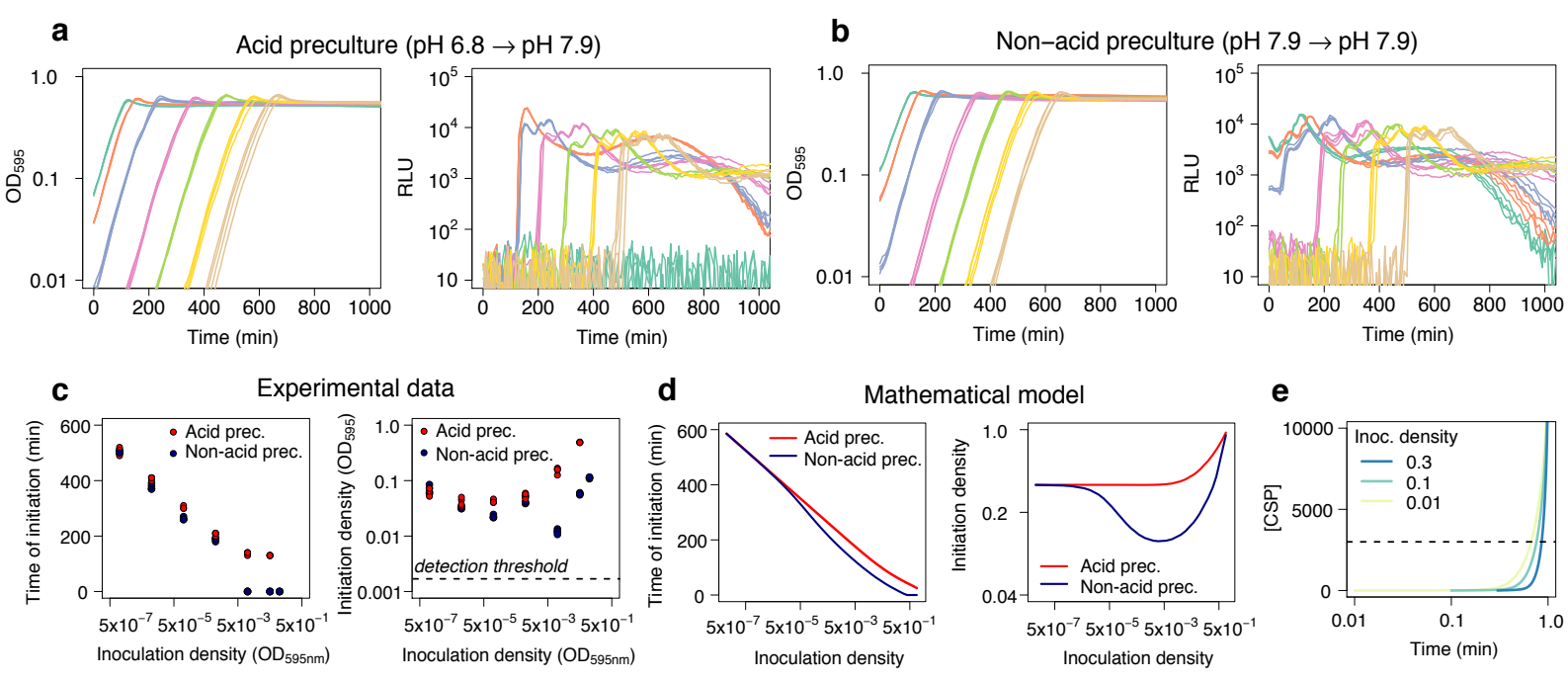
1000 **Figure 5. Competence is simultaneously regulated by cell density, pH and**
1001 **antibiotic stress.** (**a**,**b**) Predictions of the mathematical model (**a**) and experimental
1002 data (**b**) on the dependency of the time of competence initiation on inoculation density,
1003 initial pH and antibiotic stress. The x-axis in **a** corresponds to the rate of CSP export in
1004 the model, r_e, which is a proxy for pH. The color scales with the time of competence
1005 initiation with more intense red corresponding to faster development of competence.
1006 Black represents no competence development. In **b,** each box corresponds to the
1007 average initiation time of three replicates. Both the model and the experimental data
1008 show that competence develops faster at higher pH and higher inoculation densities. **c**)
1009 Antibiotics induce competence at pH values that repress natural competence
1010 development. **d**) At pH values that are not repressive for competence development (pH
1011 > 7.4), competence develops faster in the presence of antibiotics. The stars indicate
1012 which conditions are plotted in **c** and **d**. The concentrations of streptomycin and
1013 HPUra are 3 μg mL^{-1} and 0.075 μg mL^{-1}, respectively. We chose these sub-MIC
1014 concentrations to minimize the effect of antibiotic stress on growth.

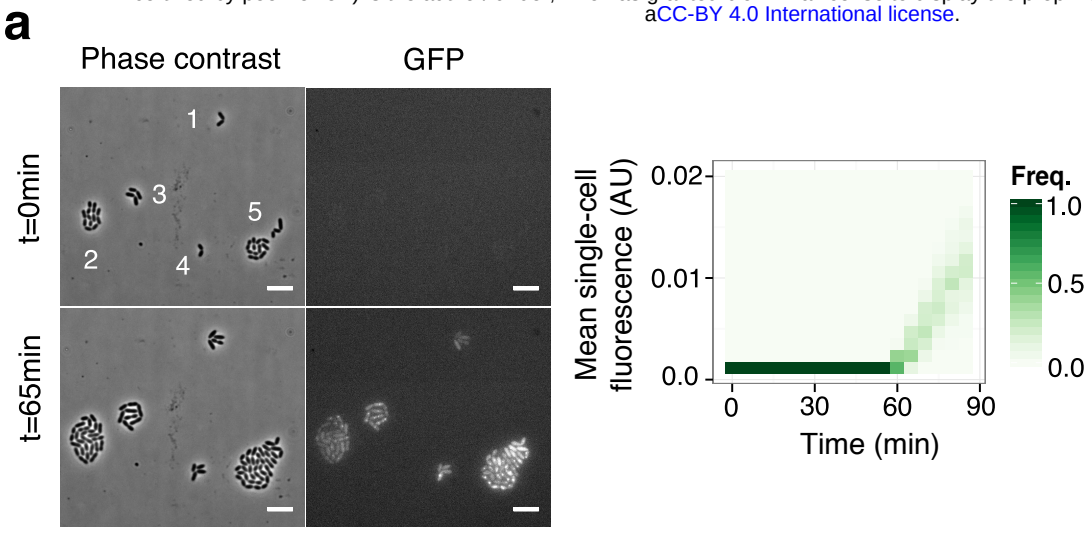
1015

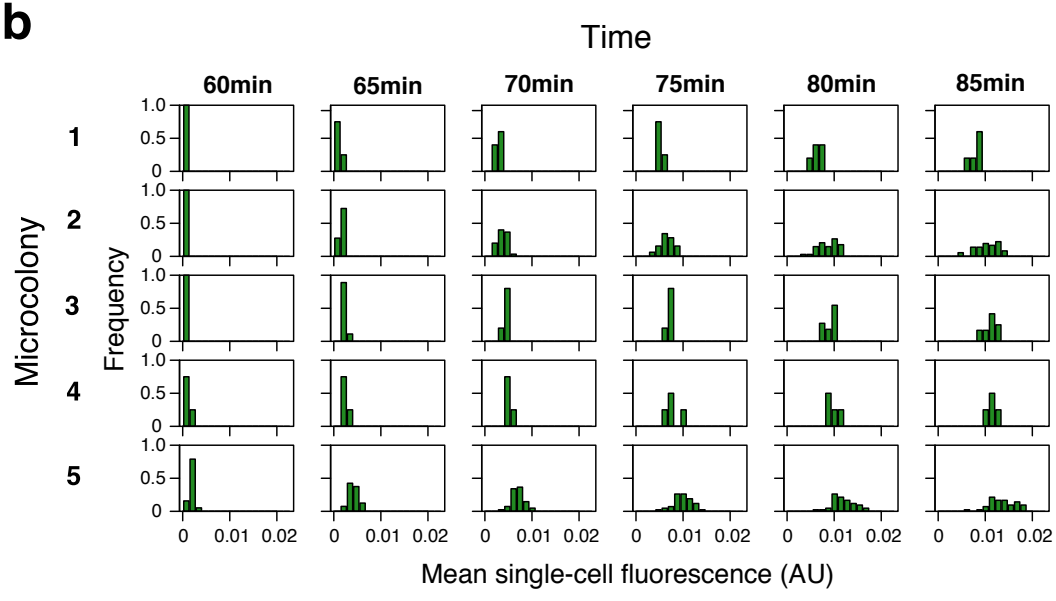
1016 **Figure 6. A bistable regime for competence development.** (**a**) Extracellular

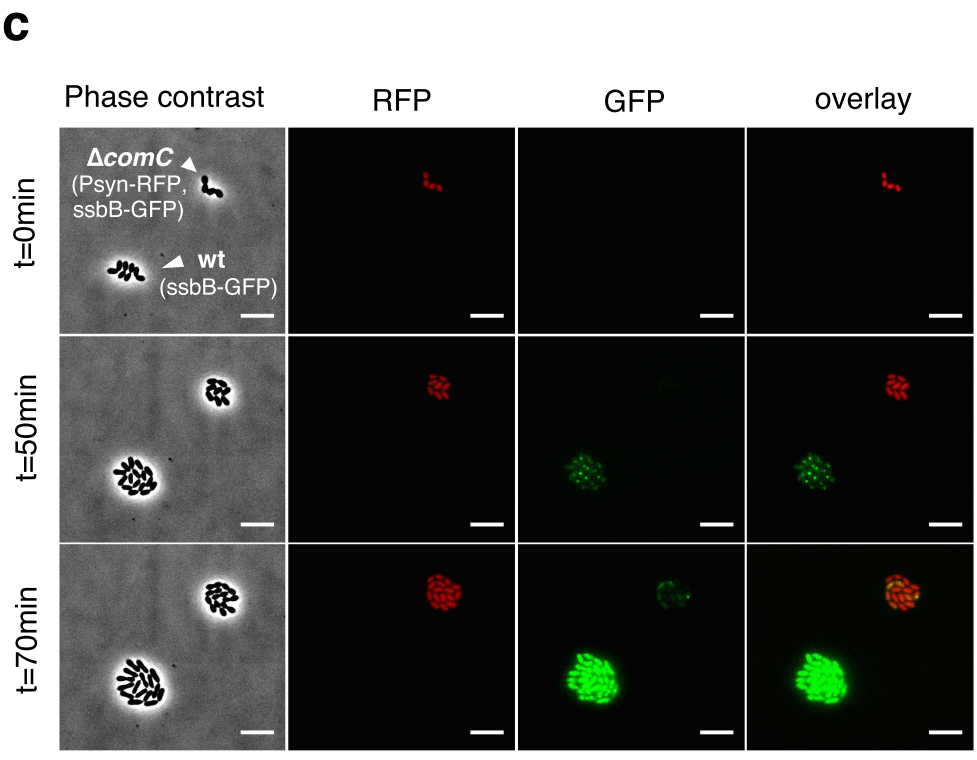
concentration of CSP in response to the rate of CSP export. The model predicts the existence of a region where competence always switches on regardless of the initial conditions (which would correspond to pH > 7.4) and of a bistable region (bordered by the dashed lines). In the latter, the initial conditions can either switch on or not CSP production and subsequently competence development. (Inset) In particular, the model predicts that in this region non-acid cell history can allow competence development if enough cells are inoculated since they can produce enough CSP to remain competent. The inoculation densities are 0.1 (Blue) and 0.01 (Brown) and both the number of ComX molecules (solid line) and the CSP concentration (dashed line) are shown. **b**) Growth curves and competence expression measured as RLU units normalized by density for cells coming from acid preculture (pH 6.8) and inoculated in medium at pH 7.4 with different initial concentrations of CSP. Three replicates are shown per treatment and all the cultures are inoculated at OD_{595} 0.002. **c**) Growth curves and competence expression measured as RLU units normalized by density for cells coming from non-acid preculture (pH 7.9) and inoculated in medium at pH 7.4 at different initial densities. Three replicates are shown per inoculation density. Competence does not develop for cells inoculated at the same densities but coming from acid preculture (pH 6.8) (Figure 5b, left panel, second column).

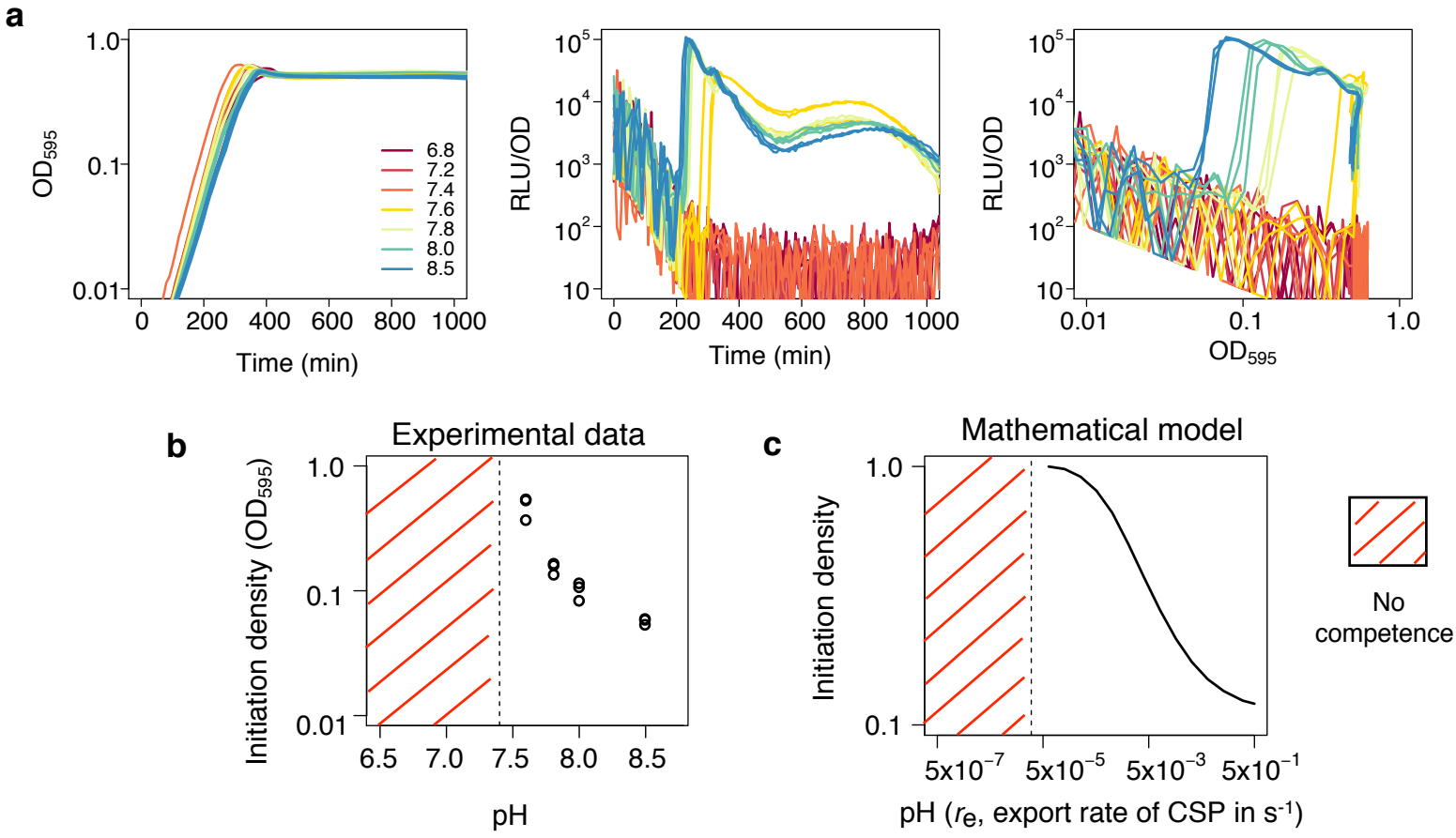


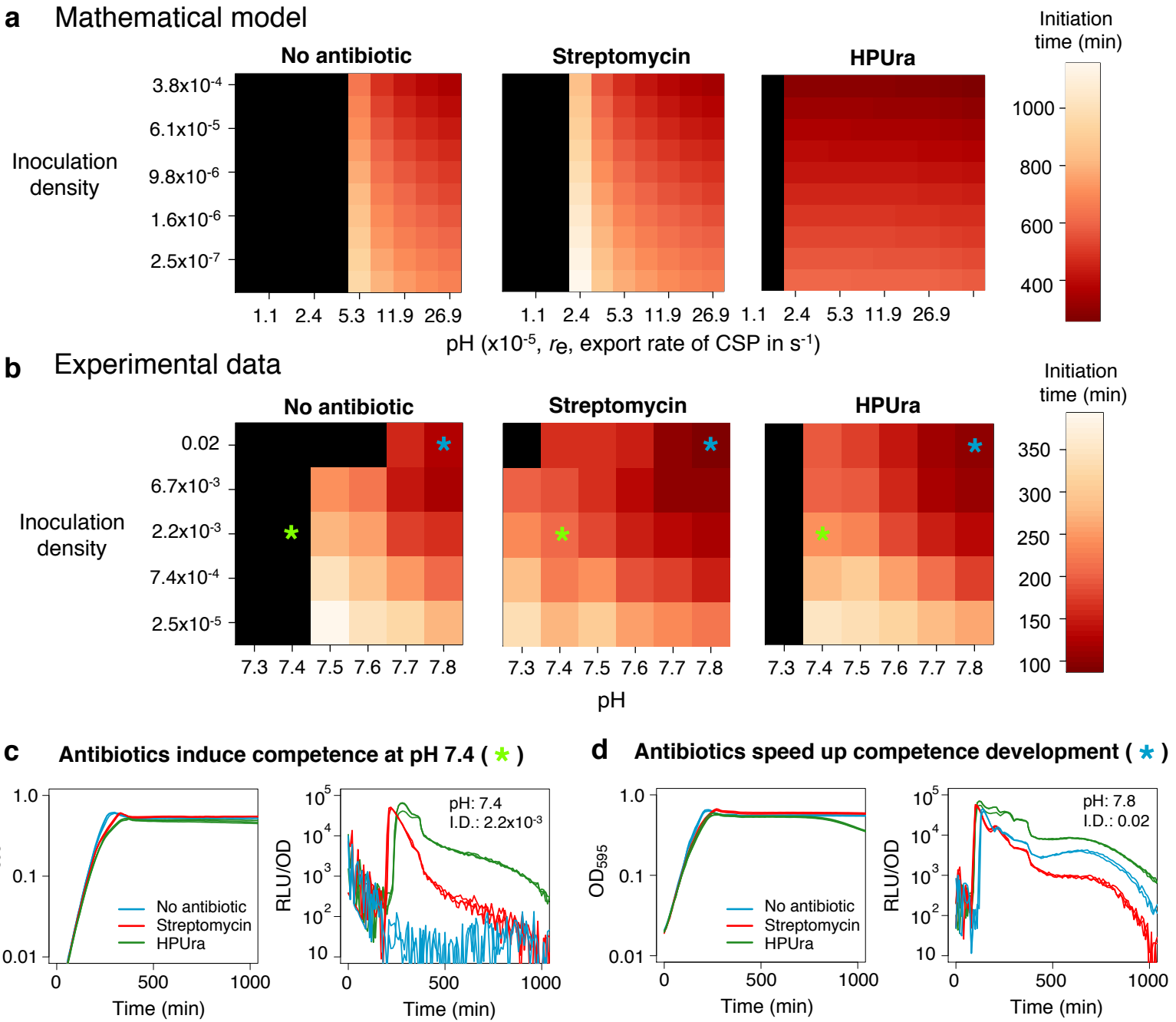

a
Acid preculture (pH 6.8 → pH 7.9)
OD595
RLU
Time (min)
b
Non−acid preculture (pH 7.9 → pH 7.9)
c
Experimental data
Time of initiation (min)
Initiation density (OD595)
Acid prec.
Non-acid prec.
detection threshold
Inoculation density (OD595nm)
d
Mathematical model
Inoculation density
e
[CSP]
Inoc. density
0.3
0.1
0.01

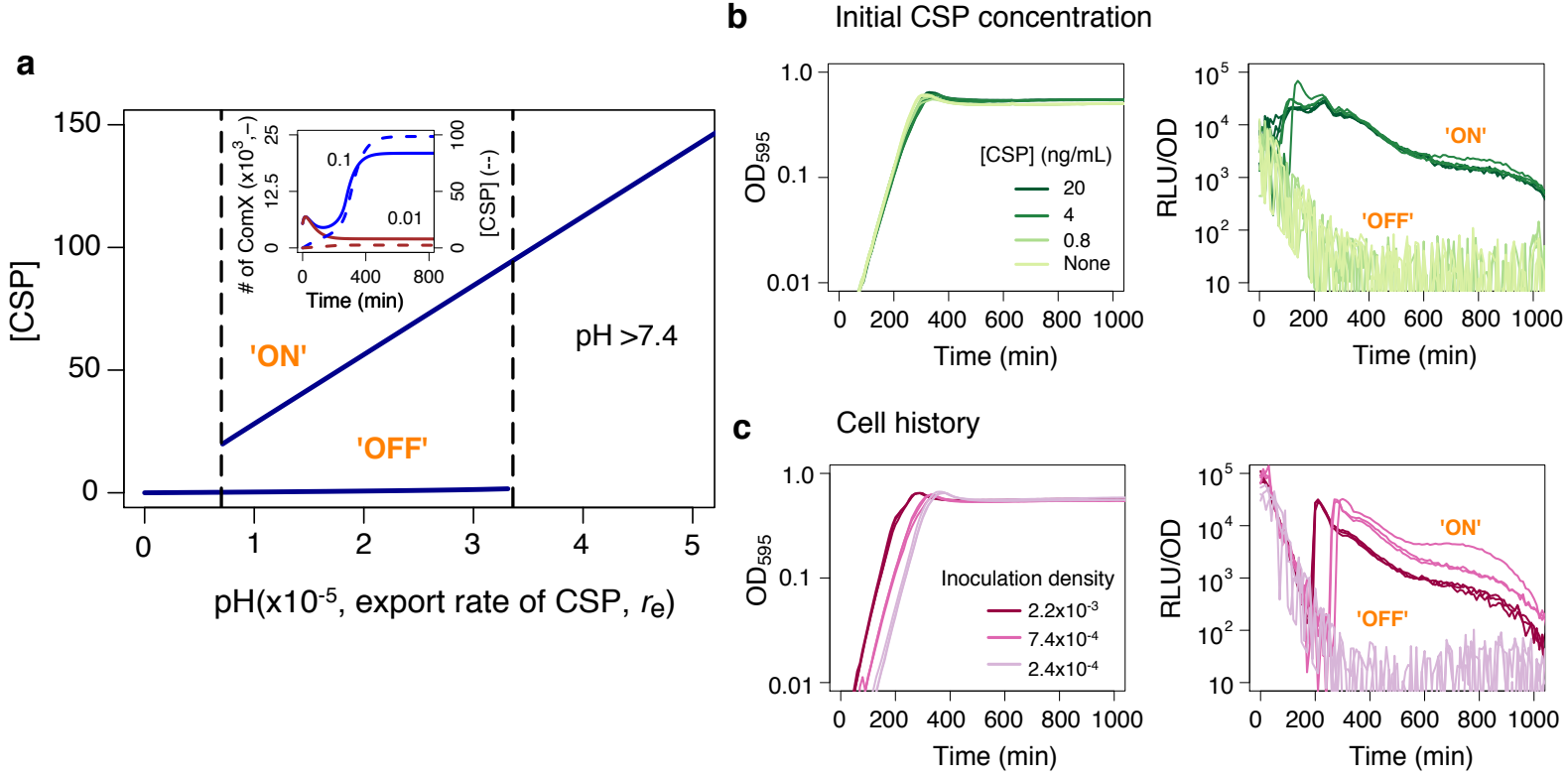







a
1.0
0.1
0.01
OD595
0
200
400
600
800
1000
Time (min)
6.8
7.2
7.4
7.6
7.8
8.0
8.5
10^5
10^4
10^3
10^2
10
RLU/OD
Time (min)
0.01
0.1
1.0
OD595
b
Experimental data
Initiation density (OD595)
6.5
7.0
7.5
8.0
8.5
pH
c
Mathematical model
Initiation density
5x10−7
5x10−5
5x10−3
5x10−1
pH (re, export rate of CSP in s-1)
No competence


a
Mathematical model
No antibiotic
Streptomycin
HPUra
Initiation time (min)
Inoculation density
3.8x10-4
6.1x10-5
9.8x10-6
1.6x10-6
2.5x10-7
1.1 2.4 5.3 11.9 26.9
1000
800
600
400
pH (x10-5, re, export rate of CSP in s-1)
b
Experimental data
No antibiotic
Streptomycin
HPUra
Initiation time (min)
Inoculation density
0.02
6.7x10-3
2.2x10-3
7.4x10-4
2.5x10-5
7.3 7.4 7.5 7.6 7.7 7.8
350
300
250
200
150
100
pH
c
Antibiotics induce competence at pH 7.4 (*)
OD595
1.0
0.1
0.01
No antibiotic
Streptomycin
HPUra
0 500 1000
Time (min)
RLU/OD
10^5
10^4
10^3
10^2
10
pH: 7.4
I.D.: 2.2x10-3
0 500 1000
Time (min)
d
Antibiotics speed up competence development (*)
OD595
1.0
0.1
0.01
No antibiotic
Streptomycin
HPUra
0 500 1000
Time (min)
RLU/OD
10^5
10^4
10^3
10^2
10
pH: 7.8
I.D.: 0.02
0 500 1000
Time (min)


a
150
100
50
0
[CSP]
0
1
2
3
4
5
pH(x10-5, export rate of CSP, re)
'ON'
'OFF'
pH >7.4
# of ComX (x10^3, –)
[CSP] (–)
0.1
0.01
Time (min)
b
Initial CSP concentration
OD595
[CSP] (ng/mL)
20
4
0.8
None
Time (min)
RLU/OD
'ON'
'OFF'
c
Cell history
Inoculation density
2.2x10-3
7.4x10-4
2.4x10-4