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

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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

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

57 We study pneumococcal competence, a system classically used as an example of
58 QS. However, whether competence is actually controlled by QS has been recently
59 debated. Competence is a transient physiological state that is developed by
60 *Streptococcus pneumoniae*, as well as other bacteria. Upon entry into competence,
61 pneumococci upregulate the expression of genes required for uptake of exogenous
62 DNA as well as bacteriocins and various genes involved in stress response [7]. In *S.*
63 *pneumoniae*, competence is regulated by an autoinducer molecule known as the
64 competence-stimulating peptide (CSP) in a two-component regulatory system formed
65 by the histidine kinase ComD and the response regulator ComE [8,9] (Figure 1). Despite
66 the detailed understanding of the regulatory network of competence induction, little is
67 known about why competence is controlled by an autoinducer peptide like CSP. CSP
68 has been classically thought to be a QS signal[10], whose function could be to monitor
69 the density of potential DNA donors[14]. However, competence can be induced in
70 response to environmental factors like pH, oxygen, phosphate and antibiotic stress [11–
71 [14]. Based on this evidence and the finding that competence initiates at the same time in

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

83 Here, we study the regulation of pneumococcal competence by cell density and two
84 environmental factors, antibiotic stress and pH. Using batch-culture experiments,
85 single cell analyses and mathematical modeling we show that these factors
86 simultaneously regulate competence development because they all affect the CSP
87 concentration: cell density sets the amount of cells producing CSP, whereas the
88 environment and cell history modify the rate at which individual cells produce or sense
89 CSP. Since there is density regulation and we show that CSP is exported
90 extracellularly, we advocate to keep using the term 'quorum sensing' in the context of
91 pneumococcal competence but with a broader meaning to acknowledge that in addition
92 to cell density, multiple factors are integrated into this QS response.
93

94 **Results**

95 **A mathematical model of pneumococcal competence development**

96 We developed a mathematical model of pneumococcal competence based on the
97 network of protein interactions known to regulate competence development (Figure 1)
98 during growth in a well-mixed liquid medium. Briefly, the precursor of CSP, ComC, is
99 cleaved and exported to the extracellular space by the membrane protein complex
100 ComAB [9,21]. Upon binding to CSP, ComD phosphorylates the response regulator
101 ComE, which in its phosphorylated form upregulates transcription of the operons
102 *comAB*, *comCDE* and *comX* [9,22,23]. The latter encodes the sigma factor ComX, which
103 controls transcription of genes required for uptake and processing of exogenous DNA
104 [7,24]. Our model uses ordinary differential equations (ODEs) and consists of two
105 components. At the population-level it keeps track of the population density and the
106 extracellular concentration of CSP; at the cell-level it keeps track of the intracellular
107 concentrations of the proteins involved in competence regulation (Figure 1). All the
108 cells export CSP to the medium at a rate determined by the intracellular concentrations
109 of ComC and ComAB. The concentration of CSP then feeds back into the intracellular
110 concentrations of all the proteins involved in competence since their transcription rates
111 depend on the ratio of ComE to ComE~P and thus on the rate at which ComD
112 phosphorylates ComE. Different environmental scenarios are simulated by changing
113 model parameters according to the known effects of such environmental factors in the
114 competence regulatory network. Since the model is primarily concerned with
115 competence initiation we purposely left out genes crucial for other aspects of
116 competence development (e.g. the stabilizing factor ComW and the immunity gene
117 *comM*) [17] and genes involved in competence shut-off such as DprA [25]. A detailed
118 description of the model and the choice of parameter values is provided in
119 Supplementary Note 1.

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

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

129

130 **Competence develops at a critical CSP concentration**

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

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

146 As shown in Figure 2a, we find that the inoculation density in strain D39 does have
147 an effect on the time of competence development, with competence initiating later for
148 lower inoculum sizes. For instance, for the lowest inoculation density, competence
149 initiates more than 4 hours later than for the highest inoculation densities (Figures 2a
150 and left panel of 2c). Note that our luminometer can detect light from competent cells
151 at an OD_{595} of 1.56×10^{-3} or higher (Supplementary Fig. 2) and therefore we cannot
152 exclude the possibility that a very small subpopulation of cells initiates competence
153 before we can detect it. Nevertheless, in all cases our estimates of the density of
154 competence initiation are far higher than the detection threshold, indicating that
155 competence in the majority of the population had not developed before crossing the
156 density threshold (Figure 2c, right panel).

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

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

175 It is well known that the pH of the medium affects competence development, with
176 natural competence being inhibited under acid conditions [11,28]. Under our experimental
177 conditions, competence only naturally develops in alkaline growth medium with a pH
178 > 7.4. So far, we have studied competence with cells precultured in a non-permissive
179 pH for competence development (pH 6.8). These preculture conditions were reflected
180 in the model simulations by assuming that cells initially were in the competence-off
181 state. We also simulated the alternative scenario that cells are already competent at
182 inoculation. For this cell history, the model predicts that the time of competence
183 initiation is lower, but only for high inoculation densities (Figure 2d, left panel). This
184 happens because when cells are competent initially and are inoculated at high density
185 they can produce enough CSP to remain competent (Supplementary Fig. 3). However,
186 when inoculation density is low, cells cannot produce enough CSP and initial
187 competence switches off. The timing of the subsequent competence initiation is then
188 the same as if cells were not competent when inoculated.

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

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

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

220 development in these mixed populations and found that competence can propagate
221 from the wildtype to the *comC*$^{-}$ mutant without the need of cell-to-cell contact (Figure
222 3C and Supplementary Movie 2). Note that the *comC*- mutant does not activate
223 competence when grown alone (Supplementary Fig. 4 and Supplementary Movies 3
224 and 4). This implies that CSP can diffuse extracellularly which confirms our previous
225 finding in liquid culture that CSP can be recovered from the supernatant of a
226 competent culture (Supplementary Fig. 1). It is worth noting that although cells release
227 CSP to an extracellular pool, a fraction might remain attached to them or in close
228 proximity due to diffusivity on the polyacrylamide surface. This can introduce
229 variation in the microenvironment that different cells experience and therefore in the
230 extent of synchronization of competence initiation.

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

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

250

251 **pH and competence development**

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

264 To study the effect of pH on competence we deleted *comC* from the *comCDE*
265 operon and put it under the control of an IPTG-inducible promoter [31] at an ectopic
266 locus. We then tested competence development in this strain at different pH values
267 (7.2, 7.4, 7.6) and at varying IPTG concentrations. For any given IPTG concentration
268 competence always developed earlier at higher pH. This indicates that cells need to
269 express more *comC* to reach the critical CSP threshold for competence activation the

270 lower the pH (Supplementary Fig. 8). In fact, at low IPTG concentrations, competence
271 only develops above pH 7.4. The same pattern is observed in an IPTG-inducible
272 *comCDE* strain: for a fixed level of *comCDE* expression, the time of competence
273 initiation still decreases with the pH (Supplementary Fig. 8). Remarkably, in this
274 genetic background competence hardly develops at pH 7.2 even when *comCDE* is fully
275 induced. Cells might need to express more *comC* at lower pH because CSP export
276 and/or detection reduces with decreasing pH. This would also explain why competence
277 barely develops at low pH in the IPTG-inducible *comCDE* strain since for a fixed level
278 of *comE* expression, reduced CSP export and/or detection would bias the
279 ComE~P/ComE ratio towards no competence development. We tested whether pH
280 affects CSP detection by using a *comC*$^{-}$ mutant. We performed experiments with
281 medium at different initial pH values where we added various concentrations of
282 synthetic CSP, using the *comC*$^{-}$ mutant. We found that competence was mainly
283 dependent on the CSP concentration and only minor differences were found among
284 media with different pH (Supplementary Fig. 8). This suggests that competence
285 development is not mainly mediated by pH-dependent CSP detection so possibly it is
286 mediated by pH-dependent export. As peptidase-containing ATP-binding cassette
287 transporters such as ComAB require ATP to transport substrates [32], it might be that the
288 proton motive force influences its activity. Therefore, we incorporated the effect of pH
289 in our model by changing the rate at which cells export CSP. In agreement with the
290 experimental results, the modified model confirms that the density of competence
291 initiation decreases with the rate of export of CSP and thus with higher pH. Also, the
292 model predicts that for rates of CSP export below a certain threshold competence does
293 not develop any more since cells never manage to accumulate enough CSP for
294 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

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

329 We reproduced the effect of HPUra and streptomycin on competence regulation in
330 our model by increasing the transcription rate of *comAB, comCDE* and *comX* and by
331 reducing the rate at which CSP degrades, respectively. Our model predicts that the
332 presence of antibiotics lowers the pH threshold for competence development (Figure
333 5a) since antibiotics can counteract the effect of acidic pH to the point that cells can
334 still accumulate enough CSP to become competent. They do this by increasing the rate
335 at which single cells produce CSP (reducing the number of cells needed to reach the
336 critical CSP concentration for competence initiation) or by increasing the rate at which
337 they sense CSP (reducing the critical CSP concentration for competence initiation).
338 Also, it predicts that for pH values where competence is already induced without
339 antibiotics, it will develop faster in the presence of antibiotics (Figure 5a). We tested
340 these predictions experimentally using antibiotics at concentrations that have a
341 minimal effect on growth since we did not incorporate growth reduction due to
342 antibiotic stress in the model. In agreement with previous studies [13,27,33] and with the
343 model predictions, we find that antibiotics can induce competence at pH values that are
344 repressive for natural competence development (Figure 5b and c). We also find support

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

358

359 **Bistable region for competence development and cell history**

360 An important feature of the competence regulatory network is the presence of a
361 positive feedback that couples CSP detection to CSP production (Figure 1). Signaling
362 systems that contain positive feedback loops often exhibit switch-like responses
363 resulting in the occurrence of alternative stable states [34]. We varied the strength of the
364 positive feedback loop in the model by changing the rate of CSP export and found that
365 the competence regulatory network exhibits bistability for a range of intermediate CSP
366 export rates. In this range, the model predicts the existence of two alternative states
367 where competence switches 'ON' or 'OFF' depending on the initial conditions (Figure
368 6a).

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

381 Bistable systems usually exhibit hysteresis. For this reason, we expected that at pH
382 7.4 where both the 'ON' and 'OFF' states are attainable, cell history, would influence
383 competence induction. From our previous experiments we determined that cells
384 coming from acid preculture inoculated at pH 7.4 do not develop competence at any
385 density of inoculation (Figure 5b left panel, second column). We then studied whether
386 there is history-dependence by inoculating cells coming from non-acid preculture at
387 pH 7.4. We found that cells coming from a non-acid preculture became competent
388 when inoculated at densities above OD_{595nm} of $2.4x10^{-4}$, which demonstrates that cell
389 history can influence competence development at this pH (Figure 6c). Past history has
390 an effect on competence because it determines the state of the machinery for CSP
391 production, which is 'OFF' when cells come from acid preculture but 'ON' when they
392 come from non-acid conditions. This explains why the effect of non-acid cell history
393 appears from a minimum inoculation density, since enough cells need to be inoculated

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

405 **Discussion**

406 Recently, the view that bacteria use autoinducers as QS signals has been debated since
407 autoinducer concentration can change in response to the environment. Here, we show
408 experimentally that cell density, pH and antibiotic stress simultaneously regulate
409 competence development in *S. pneumoniae* (Figures 2-5), a system classically framed
410 in the paradigm of QS. Using a mathematical model, we show that this occurs because
411 pH and antibiotics modify the rates at which single cells produce and sense CSP and
412 therefore the strength of the positive feedback loop coupling CSP detection to CSP
413 production (Figures 4 and 5). This environmental dependency does not override
414 regulation by cell density but rather modulates the relationship between the number of
415 cells and the CSP concentration. A fundamental aspect to the dependency on cell
416 density is that cells share CSP with others. Importantly, here we provide evidence both
417 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-

516 asparagine (331 μM), L-cysteine (71 μM), L-glutamine (150 μM), L-tryptophane
517 (29.4) μM, casein hydrolysate (5 g L^{-1}), BSA (8 mg L^{-1}), biotin (2.46 μM), nicotinic
518 acid (4.87 μM), pyridoxine (3.4 μM), calcium pantothenate (5.04 μM), thiamin (1.9
519 μM), riboflavin (0.744 μM), choline (48 μM), $CaCl_2$ (113 μM), K_2HPO_4 (48.8 mM),
520 $MgCl_2$ (2.46 mM), $FeSO_4$ (1.8 μM), $CuSO_4$ (2 μM), $ZnSO_4$ (1.74 μM), $MnCl_2$ (40
521 μM), glucose (11.1 mM), sodium pyruvate (2.7 mM), saccharose (944 μM), sodium
522 acetate (24.4 mM) and yeast extract (2.5 g L^{-1}).

523 **Construction of recombinant strains**

524 To transform *S. pneumoniae*, cells were grown in C+Y medium (pH 6.8) at 37ºC to an
525 OD_{595} of 0.1. Then, cells were treated for 12 minutes at 37ºC with synthetic CSP-1
526 (100 ng mL^{-1}) and incubated for 20 minutes at 30ºC with the transforming DNA. After
527 incubation with the transforming DNA, cells were grown in C+Y medium (pH 6.8) at
528 37ºC for 90 minutes. *S. pneumoniae* transformants were selected by plating inside
529 Columbia agar supplemented with 3% of defibrinated sheep blood (Johnny Rottier,
530 Kloosterzande, The Netherlands) and the appropriate antibiotics. Working stocks of
531 cells were prepared by growing cells in C+Y (pH 6.8) until an OD_{595} of 0.4. Cells were
532 collected by centrifugation (1595 x *g* for 10 minutes) and resuspended in fresh C+Y
533 medium (pH 6.8) with 15% glycerol and stored at -80ºC. See the details on the
534 construction of the strains below and see Supplementary Table 1 for a list of all the
535 strains used in this study.

536 ***Strain DSM2***

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

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

544 ***Strains ADP243 and ADP62***

545 To prevent the production of CSP, the original *comC* gene was replaced by an
546 erythromycin resistance marker, leaving the promoter and therefore the polycistronic
547 nature of *comDE* intact. The upstream region was amplified using primers LAG11
548 (GGCGGATCCGGCAGTTTGTGTAATAGTAC) and ADP2/48+AscI
549 (ACGTGGCGCGCCGTTCCAATTTAACTGTGTTTTTCAT), the downstream
550 region with primers ADP2/47+BamHI
551 (ACGTGGATCCGAAATAAGGGGAAAGAGTAATGGATTTATTTG) and LAG54
552 (AATCGCCATCTTCCAATCCC), and the erythromycin resistance marker with
553 0292_ery_R+BamHI (GCGGATCCTGTCTTTGACCCAATCATTC) and
554 sPG19+AscI (ACGTGGCGCGCCCGGAGGAATTTTCATATGAAC). All three
555 fragments were digested with the proper restriction enzymes (AscI and/or BamHI) and
556 ligated. The Δ*comC::ery* fragment containing the erythromycin resistance marker
557 flanked by the sequence up- and downstream of *comC* without altering the natural
558 transcription of *comDE*- was transformed into D39 and DLA3[27] resulting in strains
559 ADP243 (Δ*comC::ery*) and ADP62 (Δ*bgaA*:: P_{ssbB}-*luc*, Δ*comC::ery*), respectively.
560 Transformants were selected on Columbia blood agar containing 0.25 μg mL^{-1}
561 erythromycin. Correct deletion of *comC* was verified by PCR and sequencing.

562 ***Strains ADP49 and ADP51***

563 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

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

591 ***Strains ADP247*** *and* ***ADP248***

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

606 ***Strain ADP95***

607 To use an IPTG-inducible system, we first transformed the codon optimized *lacI* gene
608 [31] into strain DLA3 [27]. For that, we PCR-ed the fragment including *lacI* integrated into
609 the *prsA*-locus together with a gentamycin resistance cassette from chromosomal DNA
610 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

659 otherwise all experiments were started with an inoculation density of OD_{595} 0.002 with
660 cells from an acid preculture. Luciferase assays were performed in 96-wells plates with
661 a Tecan Infinite 200 PRO luminometer at 37ºC as described before [62]. Luciferin was
662 added at a concentration of 0.5 mg mL^{-1} to monitor competence by means of luciferase
663 activity. Optical density (OD_{595nm}) and luminescence (relative luminescence units
664 [RLU]) were measured every 10 minutes. The time and density of competence
665 initiation correspond to the first time point where the RLU signal is equal or above 200
666 units. RLU is used instead of RLU/OD for Figure 2 because 1) when competence
667 develops the rate at which the RLU signal increases is faster than the growth rate and
668 2) due to the very low inoculation densities used for Figure 2 the RLU/OD can be very
669 high at the start (clearly before competence has developed). The value of 200 units was
670 chosen because once this value is reached competence always developed. The effect of
671 pH on competence development was studied by inoculating cells in C+Y at a range of
672 pH values from 6.8 to 8.5. pH was adjusted by adding HCl and NaOH. The effect of
673 antibiotics was studied by adding streptomycin (3 µg mL^{-1}) and HPUra (0.075 µg mL^{-}
674 ^{1}) to C+Y.

675 **Detection of CSP in cell free supernatant**

676 *S. pneumoniae* D39 wild-type and its *comC* deficient version, ADP243, were grown in
677 non-acid C+Y (pH 7.9) at 37ºC to an OD_{595nm} of 0.1. Cells were spun down by
678 centrifugation at 20000 x g for 5 min. The supernatant was sterilized by filtering twice
679 through 0.2 µm filters. The supernatant was plated on Columbia blood agar to confirm
680 that no viable cells were present. For both the wild-type and the *comC*-deficient strain,
681 the cell-free supernatant was diluted 1:1 with 2x concentrated C+Y medium containing
682 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

707 **Data availability**

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

711

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

920 **Figure legends**

921

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

934

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

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

958

959 **Figure 3. Competence can propagate by CSP diffusion without the necessity of**
960 **cell-to-cell contact.** (**a**) Phase contrast and GFP images (left panel) of a set of
961 neighboring microcolonies of D39 with a fusion of the late competence gene *ssbB* to
962 *gfp* (P_{ssbB}-*ssbB*-*gfp*). The first images (t = 0 min) were taken right after inoculation on
963 the microscopy slide and the second ones correspond to the moment of competence
964 initiation (t = 65 min). Images were taken every 5 minutes. Crucially, when
965 competence starts the microcolonies are not in direct physical contact with each other.
966 Analysis of the fluorescence signal across the entire population (right panel) shows
967 that once competence starts, after 60 minutes from inoculation, the distribution of
968 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

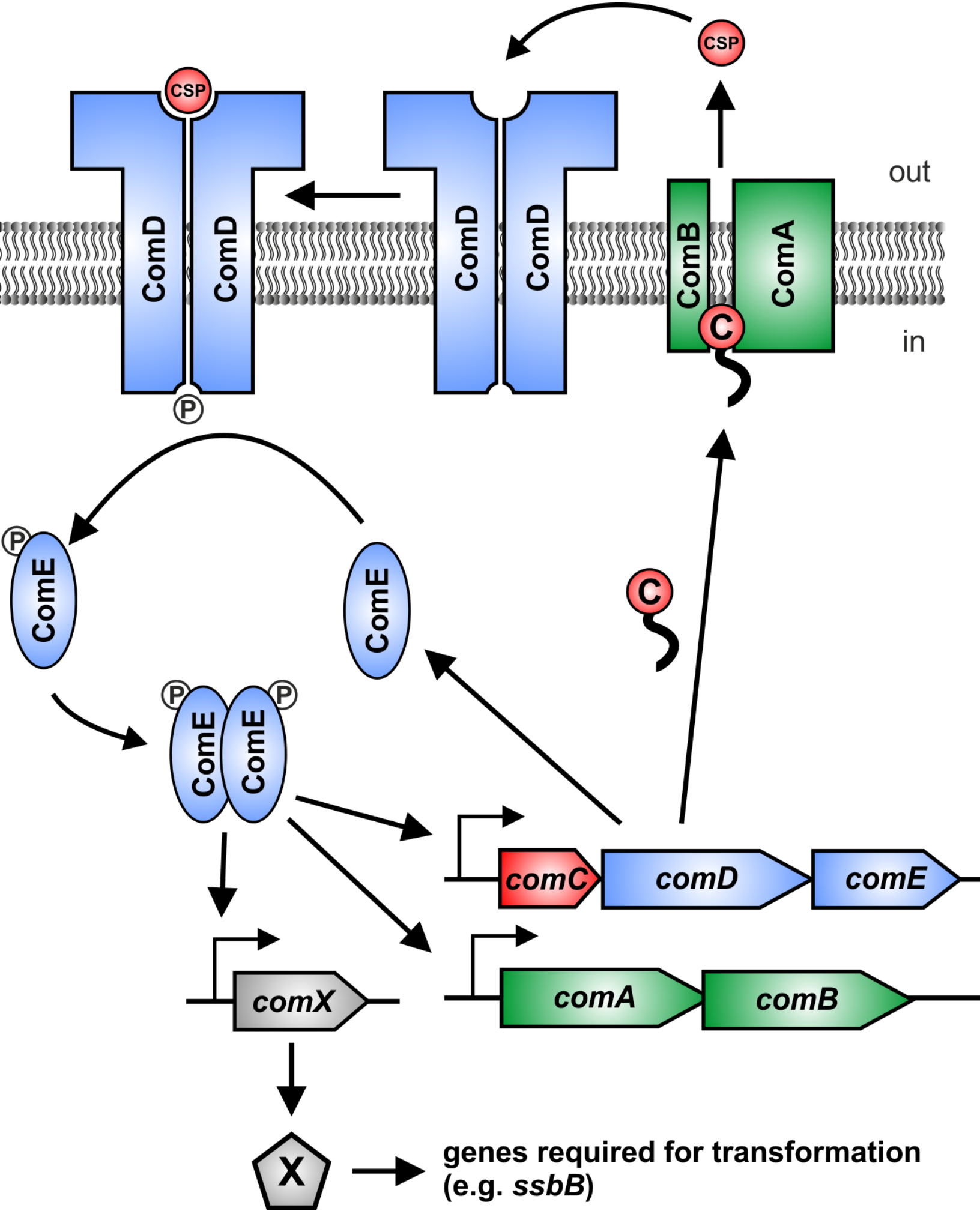
1000 **Figure 5. Competence is simultaneously regulated by cell density, pH and**
1001 **antibiotic stress.** (**a,b**) Predictions of the mathematical model (**a**) and experimental
1002 data (**b**) on the dependency of the time of competence initiation on inoculation density,
1003 initial pH and antibiotic 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 $\mu g\ mL^{-1}$ and 0.075 $\mu g\ mL^{-1}$, respectively. We chose these sub-MIC
1014 concentrations to minimize the effect of antibiotic stress on growth.

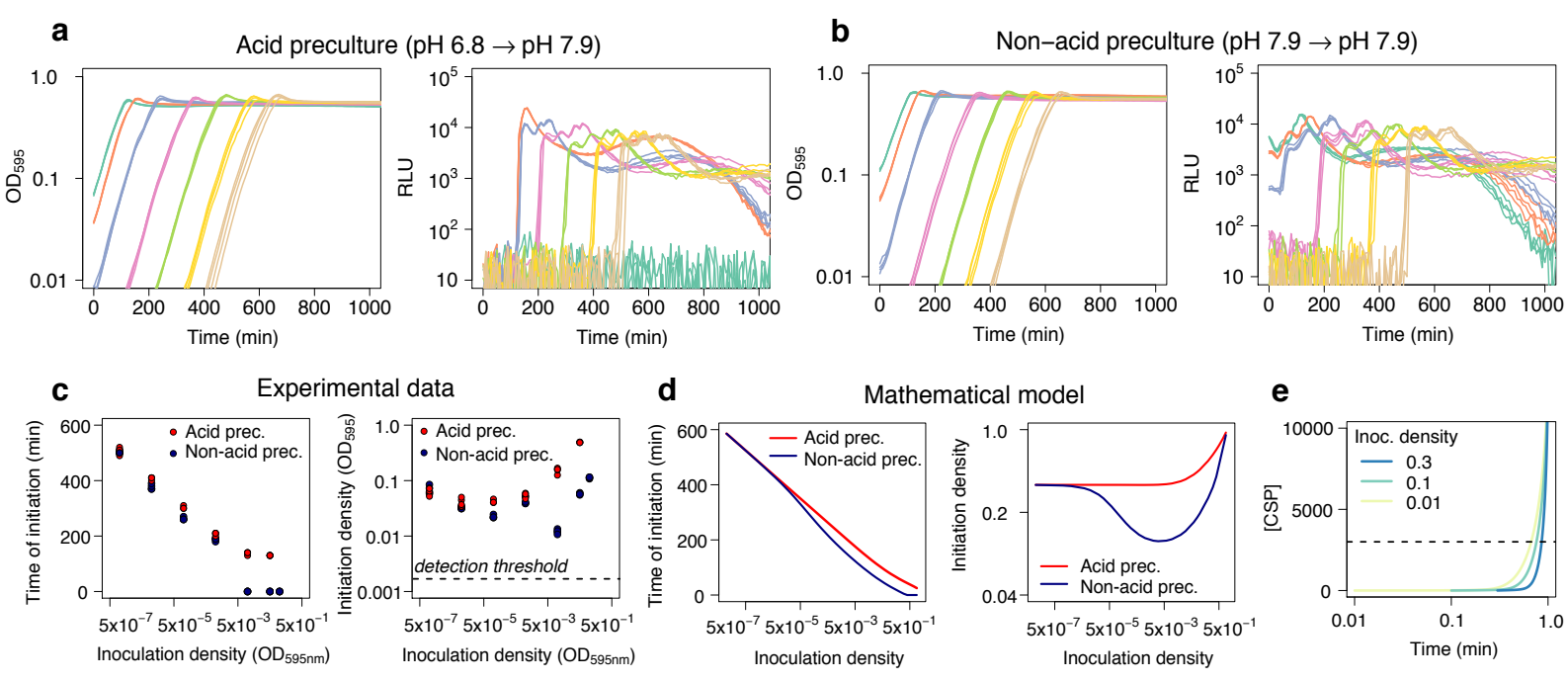
1015

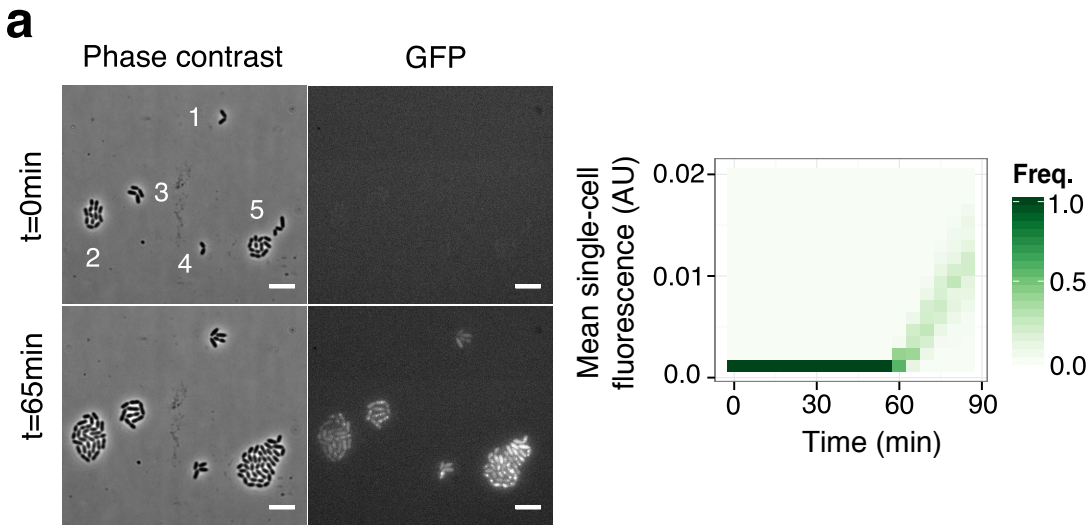
1016 **Figure 6. A bistable regime for competence development.** (**a**) Extracellular

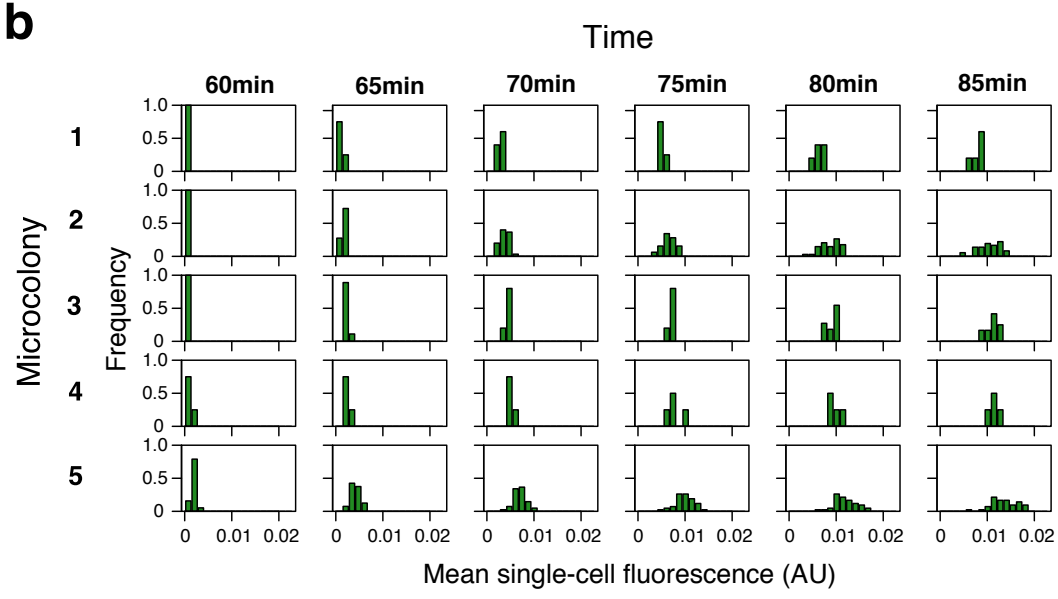
1017 concentration of CSP in response to the rate of CSP export. The model predicts the
1018 existence of a region where competence always switches on regardless of the initial
1019 conditions (which would correspond to pH > 7.4) and of a bistable region (bordered by
1020 the dashed lines). In the latter, the initial conditions can either switch on or not CSP
1021 production and subsequently competence development. (Inset) In particular, the model
1022 predicts that in this region non-acid cell history can allow competence development if
1023 enough cells are inoculated since they can produce enough CSP to remain competent.
1024 The inoculation densities are 0.1 (Blue) and 0.01 (Brown) and both the number of
1025 ComX molecules (solid line) and the CSP concentration (dashed line) are shown. **b**)
1026 Growth curves and competence expression measured as RLU units normalized by
1027 density for cells coming from acid preculture (pH 6.8) and inoculated in medium at pH
1028 7.4 with different initial concentrations of CSP. Three replicates are shown per
1029 treatment and all the cultures are inoculated at OD_{595} 0.002. **c**) Growth curves and
1030 competence expression measured as RLU units normalized by density for cells coming
1031 from non-acid preculture (pH 7.9) and inoculated in medium at pH 7.4 at different
1032 initial densities. Three replicates are shown per inoculation density. Competence does
1033 not develop for cells inoculated at the same densities but coming from acid preculture
1034 (pH 6.8) (Figure 5b, left panel, second column).

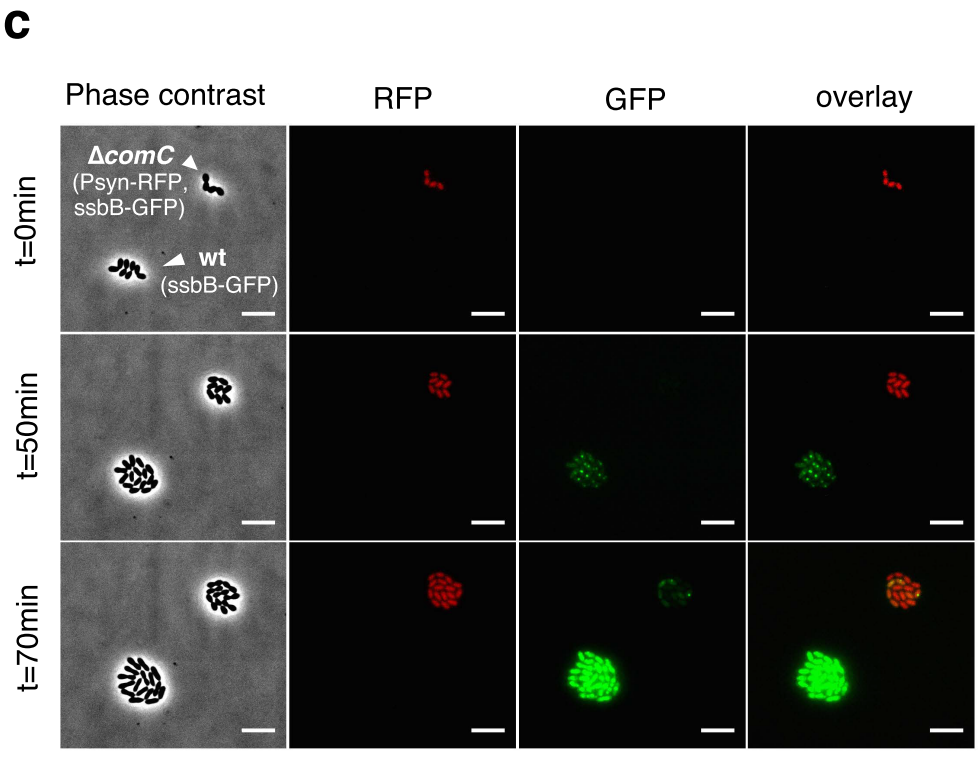
1035

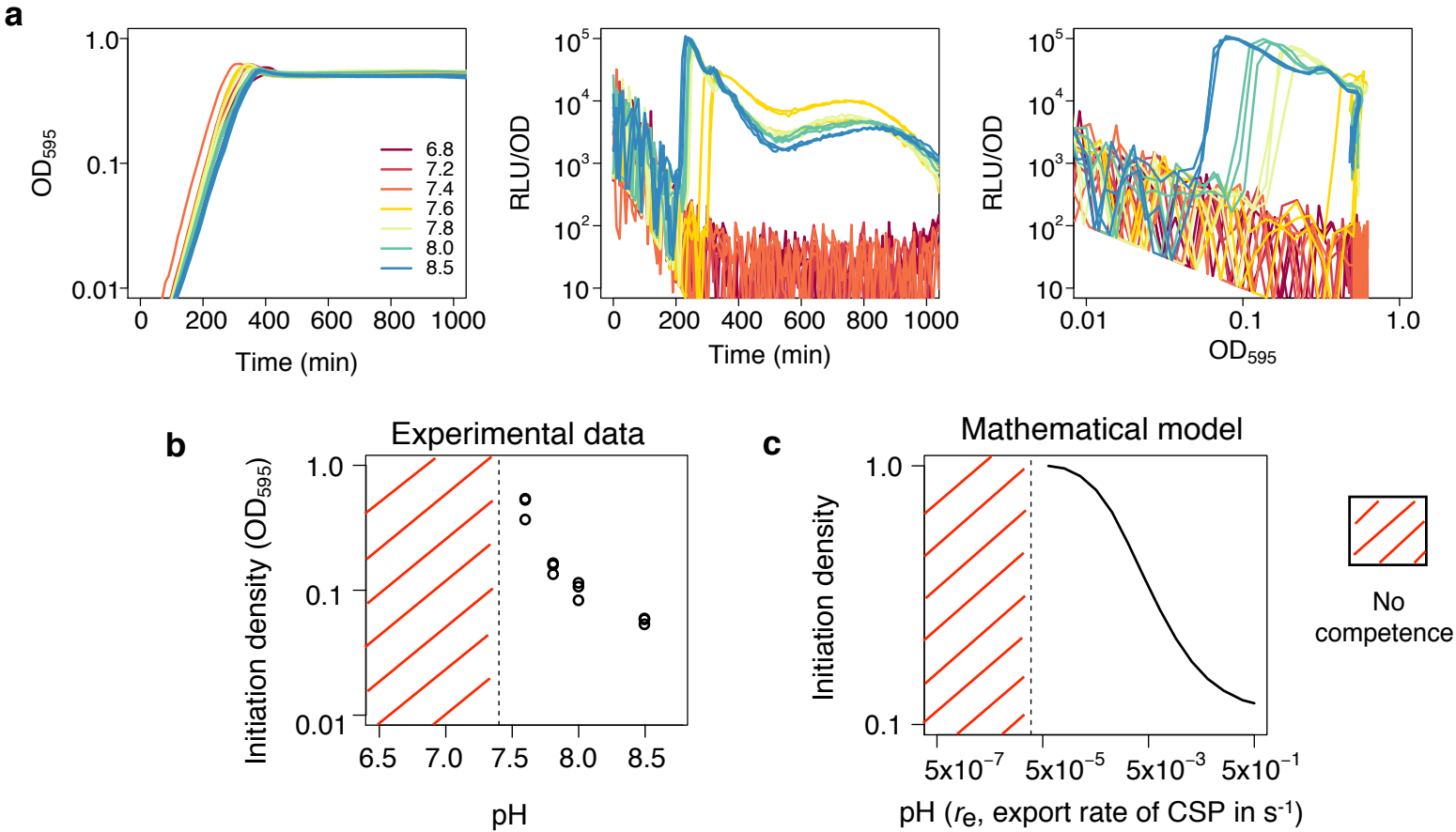

CSP
CSP
ComD
ComD
ComD
ComD
ComB
ComA
C
out
in
P
P
ComE
ComE
C
P
P
ComE
ComE
comC
comD
comE
comX
comA
comB
X
genes required for transformation
(e.g. ssbB)

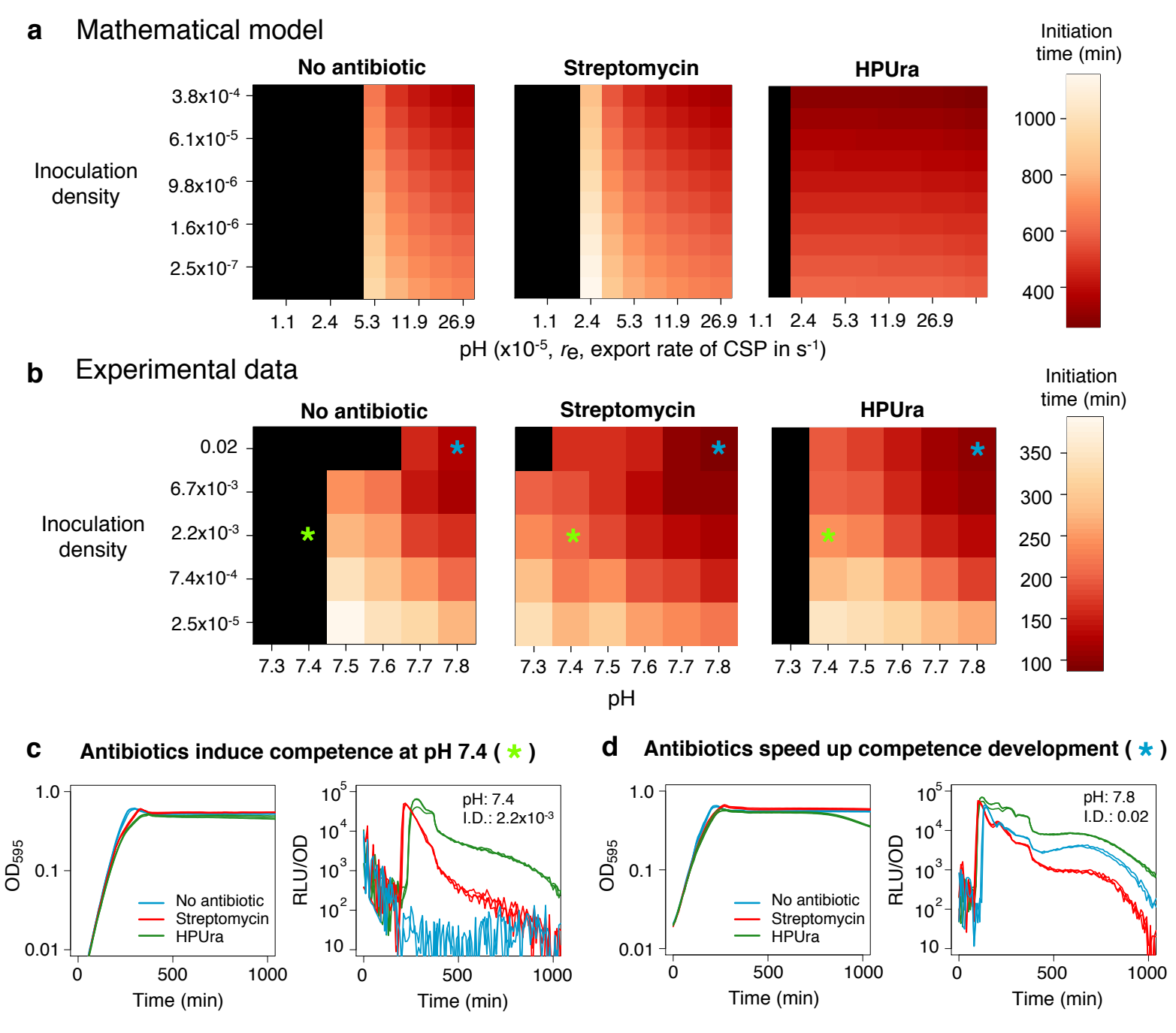

a
Acid preculture (pH 6.8 → pH 7.9)
OD595
RLU
Time (min)
b
Non–acid preculture (pH 7.9 → pH 7.9)
c
Experimental data
Acid prec.
Non-acid prec.
Time of initiation (min)
Initiation density (OD595)
detection threshold
Inoculation density (OD595nm)
d
Mathematical model
Initiation density
e
Inoc. density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

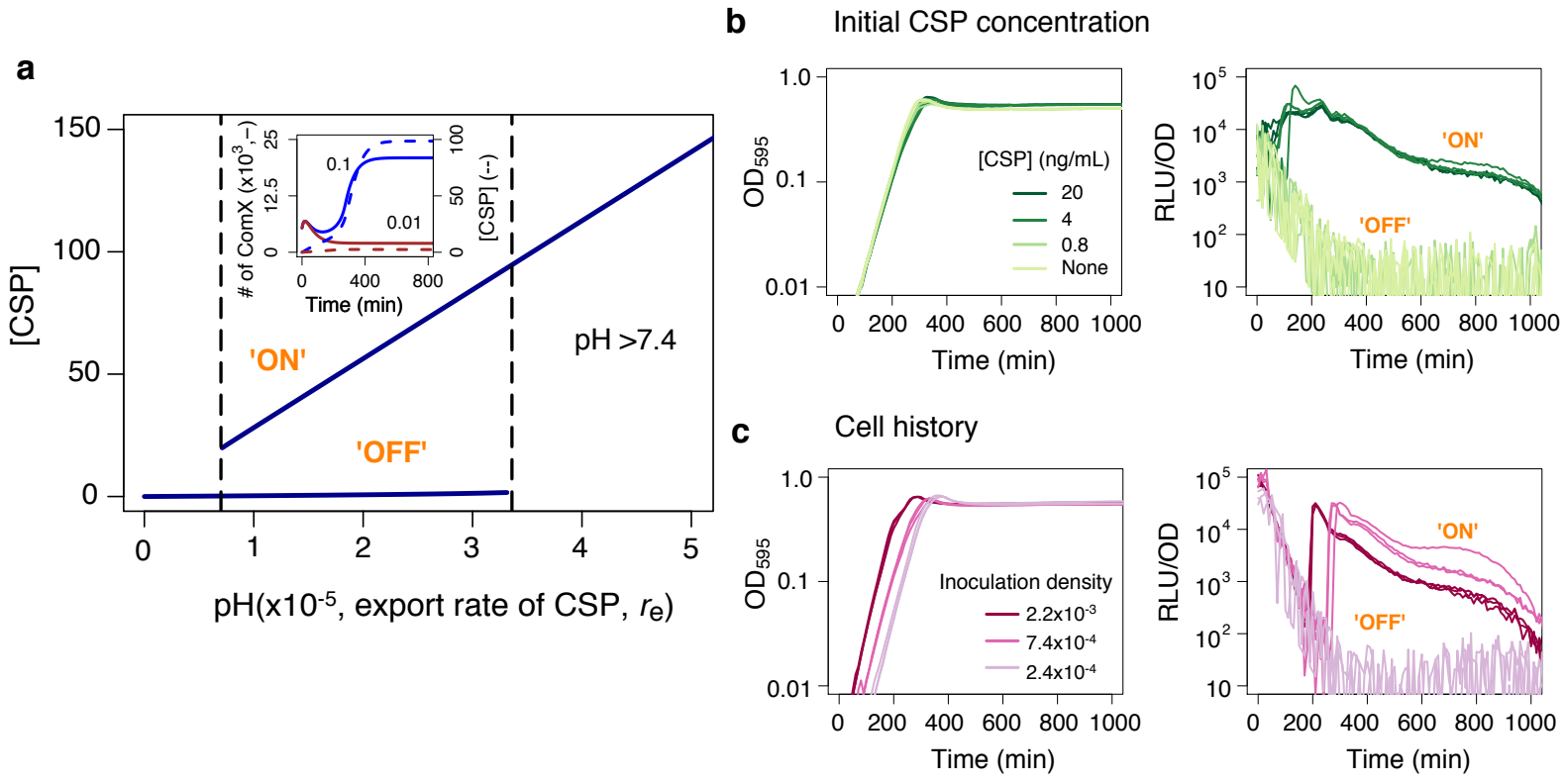

a
Phase contrast
GFP
t=0min
t=65min
1
2
3
4
5
Mean single-cell fluorescence (AU)
0.02
0.01
0.0
0
30
60
90
Time (min)
Freq.
1.0
0.5
0.0


b
Time
60min
65min
70min
75min
80min
85min
Microcolony
1
2
3
4
5
Frequency
1.0
0.5
0
0.01
0.02
Mean single-cell fluorescence (AU)


c
Phase contrast
RFP
GFP
overlay
t=0min
t=50min
t=70min
ΔcomC
(Psyn-RFP,
ssbB-GFP)
wt
(ssbB-GFP)


a
OD595
1.0
0.1
0.01
0
200
400
600
800
1000
Time (min)
6.8
7.2
7.4
7.6
7.8
8.0
8.5
RLU/OD
10^5
10^4
10^3
10^2
10
Time (min)
RLU/OD
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
[CSP]
150
100
50
0
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
1.0
0.1
0.01
[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