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

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Summary

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 individuals 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 depends simultaneously on 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 a reliable 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 also 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

46 expressed by many cells [1,2]. However, it is likely that the concentration of autoinducer
47 molecules does not only reflect cell density, but that it is also affected by
48 environmental factors, such as the diffusivity of the medium. In fact, alternative
49 hypotheses state that bacteria release autoinducers to sense these environmental factors
50 rather than to monitor cell density. The most well known hypothesis proposed by
51 Redfield (2002) is that the function of autoinducers is diffusion sensing, allowing cells
52 to avoid the secretion of costly molecules under conditions where they would quickly
53 diffuse away [3]. Evidence for the role of environmental diffusion on the concentration
54 of autoinducers comes from experiments showing that when diffusion is strongly
55 limited, a small group of cells (or even a single cell) can upregulate gene expression by
56 producing autoinducers [4–6]. Other potential roles suggested for autoinducer production
57 are sensing local cell density together with diffusion [7], the positioning of other cells
58 during biofilm formation [8] and temporal variations in pH [9].

59 We study pneumococcal competence, a system classically used as an example of
60 QS whose functional role has been recently debated in light of the previous
61 controversies. Competence is a transient physiological state that is developed by
62 *Streptococcus pneumoniae*, as well as other bacteria. Upon entry into competence,
63 pneumococci upregulate the expression of genes required for uptake of exogenous
64 DNA as well as bacteriocins and various genes involved in stress response [10]. In *S.*
65 *pneumoniae*, competence is regulated by an autoinducer molecule known as the
66 competence-stimulating peptide (CSP) in a two-component regulatory system formed
67 by the histidine kinase ComD and the response regulator ComE [11,12] (Figure 1).
68 Despite the detailed understanding of the regulatory network of competence induction,
69 little is known about why competence is controlled by an autoinducer peptide like
70 CSP. Although CSP has been classically thought to be a QS signal [13], competence can

71 be induced in response to environmental factors like pH, oxygen, phosphate and
72 antibiotic stress [14–17]. For instance, under our experimental conditions, competence
73 only naturally develops in alkaline growth medium with a pH > 7.4 (see below). Based
74 on this evidence and the finding that competence initiates at the same time in
75 pneumococcal cultures inoculated at different initial densities, it was suggested that
76 CSP acts as a timing device that allows cells to mount a timed response to
77 environmental stress independently of cell density [18,19]. Since then, this hypothesis has
78 established in the field as an alternative to the QS view of competence [16,20,21,19].
79 Recently, Prudhomme et al. (2016) renamed the timing device mechanism as a growth-
80 time dependent mechanism and proposed that a subpopulation of competent cells that
81 originates stochastically spreads the competent state to the rest of the population by
82 cell-cell contact [22]. Another alternative to QS is that pneumococcal competence is an
83 instance of diffusion sensing. This was suggested by Yang et al. (2010) based on the
84 observation that the quorum for competence induction is not fixed but decreases with
85 more restrictive diffusion [23].

86 Here, we study the regulation of pneumococcal competence by cell density and two
87 environmental factors, antibiotic stress and pH. Using both experiments and
88 mathematical modeling we study the combined action of these factors on competence
89 induction. We make predictions based on the model regarding the time and density at
90 which competence initiates under different combinations of inoculation densities, cell
91 histories and environmental conditions and show that they are confirmed by
92 experimental data. A common observation across environmental contexts is that the
93 concentration of CSP always increases with the number of cells, supporting the
94 functional interpretation of competence as a QS system; however, we also find that the
95 exact relationship between cell density and CSP concentration is determined by

environmental factors and cell history which modify the rate at which individual cells produce or sense CSP. Based on these findings, we advocate to keep using the term 'quorum sensing' but with a broader meaning to acknowledge that in addition to cell density, multiple factors regulate autoinducer production and detection. This perspective emphasizes the importance of considering the natural context where bacteria use QS when formulating hypotheses on the functional role of responding to any particular biotic or abiotic factor through QS.

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 [12,24]. Upon binding to CSP, ComD phosphorylates the response regulator ComE, which in its phosphorylated form upregulates transcription of the operons *comAB*, *comCDE* and *comX* [12,25,26]. The latter encodes the sigma factor ComX, which controls transcription of genes required for uptake and processing of exogenous DNA [10,27]. 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

121 depend on the ratio of ComE to ComE~P and thus on the rate at which ComD
122 phosphorylates ComE. The model uses changes in competence gene expression and/or
123 changes in the rate at which cells export CSP to simulate different environmental
124 scenarios (see below). A detailed description of the model and the choice of parameter
125 values is in the Supplementary Information.

126 We use the model to determine the effect of environmental factors and cell
127 history on the relationship between cell density and CSP concentration. Crucially, the
128 model assumes that all cells are homogeneous and that competence is only regulated
129 by CSP, whose production increases with cell density. We are interested in
130 determining whether these assumptions are sufficient to explain our experimental
131 results or if additional mechanisms need to be incorporated (e.g. density-independent
132 competence induction and stochastic induction of competence in a subpopulation of
133 cells [18,19,22]).

134

135 **Competence is regulated by cell density and develops at a critical CSP**
136 **concentration**

137 It has been reported that competence develops at a fixed time after inoculation from
138 acid to alkaline conditions (pH 6.8 → 7.9) regardless of the inoculum size [18,22]. This
139 observation has motivated the view that competence develops independently of cell
140 density and rather acts as a timed response at the single cell level to the pH shift
141 occurring at the moment of inoculation. We extended previous studies by exploring a
142 wider range of inoculation densities (OD_{595nm}: 10^{-1} – 10^{-7}) (approximately 10^{7} – 10^{1}
143 cells/ml) and preculturing conditions. We used the encapsulated serotype 2 strain *S.*
144 *pneumoniae* D39 [28] and cells were washed before inoculation to remove CSP produced

145 during the preculture (note that we verified that CSP is actually present in the
146 supernatant of cultures of strain D39; see Figure S1). To monitor competence
147 development, the ComX-dependent promoter of the late competence gene *ssbB* was
148 fused to the firefly *luc* gene and inserted at the non-essential *bgaA* locus. Activation
149 and expression of *ssbB* is a good reporter for competence development since SsbB
150 expression strongly correlates with actual transformation with externally added DNA
151 (e.g. [16,29] and data not shown).

152 As shown in Figure 2a, and in contrast to Claverys *et al.* (2006)[18], we find that the
153 inoculation density does have an effect on the time of competence development, with
154 competence initiating later for lower inoculum sizes. For instance, for the lowest
155 inoculation density, competence initiates more than 4 hours later than for the highest
156 inoculation densities (Figures 2a and left panel of 2c). Importantly, the population
157 density at competence initiation is not constant but positively related to the inoculation
158 density (Figure 2c, right panel). Hence, the dependency of the time of competence
159 initiation on the inoculation density is not a consequence of competence developing at
160 a fixed critical cell density for every condition. Instead, our results are consistent with
161 the mathematical model, which predicts that competence develops when the CSP
162 concentration has reached a critical threshold. The model shows that competence will
163 start faster for higher inoculation densities because the CSP concentration reaches the
164 critical threshold for competence activation earlier if more cells are producing CSP
165 (Figure 2d, left panel). Moreover, the model shows that populations inoculated at low
166 densities initiate competence at a lower density than populations inoculated at high
167 densities consistent with the experimental data (right panels of Figures 2c and 2d).
168 This is because cells inoculated at low cell densities already had time to start
169 transcribing competence regulatory genes and accumulate some CSP once they

170 reached the same cell density of cultures freshly inoculated at a higher cell density
171 (Figure 2e). Thus, the critical CSP threshold is reached sooner for low-density
172 inoculated cultures. Notably, a common misconception in the field is that in a QS
173 system the critical concentration of autoinducer should always be attained at the same
174 fixed cell density [18,19,22,23].

175 It is well known that the pH of the medium affects competence development, with
176 natural competence being inhibited under acid conditions [14,30]. So far we have studied
177 competence with cells precultured in a non-permissive pH for competence
178 development (pH 6.8). These preculture conditions were reflected in the model
179 simulations by assuming that cells initially were in the competence-off state. We also
180 simulated the alternative scenario that cells are already competent at inoculation. For
181 this cell-history, the model predicts that the time of competence initiation is lower, but
182 only for high inoculation densities (Figure 2d, left panel, Figure S2). This happens
183 because when cells are competent initially and are inoculated at high density they can
184 produce enough CSP to remain competent. However, when inoculation density is low,
185 cells cannot produce enough CSP and initial competence switches off. The timing of
186 the subsequent competence initiation is then the same as if cells were not competent
187 when inoculated (Figure S3).

188 To verify the predicted effect of cell-history on the timing of competence initiation,
189 we controlled the competence state of cells at inoculation by manipulating the pH
190 during preculture. Specifically, we compared the time of competence initiation for
191 cells coming from a non-permissive (pH 6.8) and a permissive (pH 7.9) pH history for
192 competence development. For inoculation densities below OD_{595nm} 0.01, the pH of the
193 preculture did not have an effect on the timing of competence initiation (Figures 2b
194 and c). On the other hand, for inoculation densities above OD_{595nm} 0.01, there was a

195 time delay in competence initiation for cells with an acid history whereas cells with a
196 non-acid history were competent when inoculated and remained competent afterwards
197 as predicted by the model (Figures 2b and d). This suggests that when the inoculation
198 density is high, there are enough cells to take the CSP concentration above the
199 threshold for competence activation if they are already producing CSP – as is the case
200 of cells coming from a non-acid history. By contrast, if cells come from a non-
201 permissive pH for competence development, the machinery for CSP production needs
202 to be activated even for the high cell densities. This causes a delay in competence
203 initiation, which has been interpreted as evidence suggesting that competence acts as a
204 timing device independently of cell density [18,19].

205 To further corroborate the effect of cell density on competence, we studied
206 competence initiation at the single-cell level in populations inoculated at two different
207 densities. The *ssbB* promoter was fused to GFP and competence initiation was
208 followed using automated fluorescence time-lapse microscopy. In line with the results
209 from liquid cultures, we find that competence initiated earlier for the highest
210 inoculation density (Figure 3). Importantly, we observe little cell-to-cell variation in
211 the time of competence initiation and found no evidence of strong phenotypic
212 heterogeneity, as is for instance observed during competence development in *Bacillus*
213 *subtilis* (where less than 20% of the population enters the competent state) [31,32]. This
214 justifies the model assumption that the population of cells is homogeneous and argues
215 against the hypothesis that a competent subpopulation emerges before the entire
216 population becomes competent [22].

217

218 **pH and competence development**

219 In order to understand how environmental factors affect competence we quantified the
220 effect of external pH on natural competence development. We studied competence at a
221 fine-grained range of pH values from 6.8 to 9.0 and found a clear-cut value that
222 separated permissive from non-permissive external pH values for natural competence
223 development as reported before [14,29]. For our media this was pH 7.4 (Figure 4a).
224 However, not only competence always developed at pH higher than 7.4 but the critical
225 cell density for competence initiation decreased with increasing pH (Figure 4a and b).
226 Therefore, pH does not relate to competence as a binary permissive/non-permissive
227 condition but competence development is more efficient in more alkaline media. The
228 data suggests that for non-permissive pH conditions the cell density at which
229 competence would initiate is above the carrying capacity of the medium, which was
230 also previously proposed by Chen and Morrison (1987)[14].

231 To test whether pH mainly affects CSP production or detection, we used a *comA*$^-$
232 mutant that is unable to export CSP and therefore only develops competence in the
233 presence of external CSP (Figure 1). We then performed experiments with medium at
234 different initial pH values where we added various concentrations of synthetic CSP,
235 using both the wild type and the *comA*$^-$ mutant. We found that for the *comA*$^-$ mutant
236 competence was mainly dependent on the CSP concentration and only minor
237 differences were found among media with different pH. For the wild type, in contrast,
238 the minimum CSP concentration required for competence development varied with the
239 pH (Figure S4). The finding that pH strongly affects competence initiation in the wild
240 type but not in the *comA*$^-$ mutant suggests that competence development is mediated
241 mainly by pH-dependent CSP secretion rather than by pH-dependent CSP detection.
242 As peptidase-containing ATP-binding cassette transporters such as ComAB require

243 ATP to transport substrates [33], it might be that the proton motive force influences its
244 activity. Therefore, we incorporated the effect of pH in our model by changing the rate
245 at which cells export CSP. In agreement with the experimental results, the modified
246 model confirms that the density of competence initiation decreases with the rate of
247 export of CSP and thus with higher pH. Also, the model predicts that for rates of CSP
248 export below a certain threshold competence does not develop any more since cells
249 never manage to accumulate enough CSP for competence to initiate (Figure 4c). Note
250 however that this is a simplification of the effect of pH in competence regulation since
251 pH might also affect ComD and/or the stability of CSP (as in other QS systems [9]) and
252 as our data suggest it might also be involved in the shutdown of competence (Figure
253 S4). However, regardless of the exact mechanism, as long as higher pH increases the
254 rate at which single cells produce and/or sense CSP, our model predicts that the density
255 at which the critical CSP concentration for competence activation is attained will
256 decrease with increasing pH.

257 Finally, we assessed the joint effect of pH and cell density on competence
258 regulation. We did this by studying competence initiation for cultures inoculated at
259 different cell densities in media with different pH both experimentally and using the
260 model. The model predicts that competence will initiate earlier both for higher
261 inoculation densities and more alkaline pH (Figure 5a left panel): While higher
262 inoculation densities mean that more cells will start producing CSP after inoculation,
263 higher pH increases the rate at which individual cells produce CSP. The experimental
264 data is consistent with this prediction (Figure 5b left panel). Therefore, the observation
265 that pH affects competence development is not conflicting with regulation by cell
266 density because the CSP concentration depends on both of these factors.

267

268 **Induction of competence by antibiotics**

269 The induction of pneumococcal competence is affected by the presence of certain
270 classes of antibiotics [16,29], which has been considered additional evidence for the
271 hypothesis that competence can be regulated independently of cell density [16,18]. We
272 evaluated this claim by studying the role of HPUra and streptomycin on competence
273 regulation. We chose these antibiotics since the mechanisms by which they induce
274 competence at the molecular level have been elucidated to some extent: HPUra stalls
275 replication forks during DNA replication while initiation of DNA replication
276 continues, thereby increasing the copy number of genes near the origin of replication
277 (*oriC*). As a consequence, it up-regulates transcription of *comAB, comCDE* and *comX*
278 as these operons are located proximal to *oriC* [29]. Streptomycin causes mistranslation
279 and is thought to regulate competence via the membrane protease HtrA which targets
280 misfolded proteins and also represses competence possibly by degrading CSP [34] (also
281 see Figure S5). By increasing the amount of misfolded proteins, streptomycin could
282 reduce the rate at which CSP is degraded by HtrA leading to competence induction.

283 We reproduced the effect of HPUra and streptomycin on competence regulation in
284 our model by increasing the transcription rate of *comAB, comCDE* and *comX* and by
285 reducing the rate at which CSP degrades, respectively. Our model predicts that the
286 presence of antibiotics lowers the pH threshold for competence development (Figure
287 5a) since antibiotics can counteract the effect of acidic pH to the point that cells can
288 still accumulate enough CSP to become competent. They do this by increasing the rate
289 at which single cells produce CSP (reducing the number of cells needed to reach the
290 critical CSP concentration for competence initiation) or by increasing the rate at which
291 they sense CSP (reducing the critical CSP concentration for competence initiation).
292 Also, it predicts that for pH values where competence is already induced without

293 antibiotics, it will develop faster in the presence of antibiotics (Figure 5a). In
294 agreement with previous studies [16,29,34] and with the model predictions, we find that
295 antibiotics can induce competence at pH values that are repressive for natural
296 competence development (Figure 5b and c). We also find support for the second
297 prediction of the model since for permissive pH values for natural competence
298 development (above 7.4), competence is induced earlier in the presence of antibiotics
299 (Figure 5b and d). Remarkably, both the model and the experiments show that the
300 combined effect of pH and cell density in the presence of antibiotics remains the same
301 as when no antibiotics are added (compare left panel with middle and right panels in
302 Figures 5a and 5b): Competence induction still occurs earlier for high densities of
303 inoculation and more alkaline pH values. In the case of HPUra at pH 7.3 it is even
304 possible to see that competence does not develop for the highest inoculation density as
305 the population probably reaches carrying capacity before enough CSP is produced.

306

307 **Bistable region for competence development and the role of cell history**

308 An important feature of the competence regulatory network is the presence of a
309 positive feedback that couples CSP detection to CSP production (Figure 1). Signaling
310 systems that contain positive feedback loops often exhibit switch-like responses
311 resulting in the occurrence of alternative stable states [35]. We varied the strength of the
312 positive feedback loop in the model by changing the rate of CSP export and found that
313 the competence regulatory network exhibits bistability for a range of intermediate CSP
314 export rates. In this range, the model predicts the existence of two alternative states
315 where competence switches 'ON' or 'OFF' depending on the initial conditions (Figure
316 6a).

317 Since in the model the rate of CSP export is positively correlated to the pH, we
318 expected to find a region of pH values exhibiting similar bistability as an additional
319 experimental corroboration of the model. Indeed, we found support for the existence of
320 a bistable region at pH 7.4 where the wild type developed competence if CSP was
321 externally added in concentrations above 4 ng mL^{-1} (Figure 6b). Thus, whereas
322 competence always switched on for pH values above 7.4 regardless of the initial CSP
323 concentration, for pH 7.4 both 'ON' and 'OFF' states were observed depending on the
324 initial CSP concentration. Moreover, at pH 7.4 competence developed for CSP
325 concentrations that did not induce competence in the *comA*$^{-}$ mutant (Figure S4), which
326 indicates that CSP production in the wild type was kick-started by the initial addition
327 of CSP resulting in enough overall CSP for competence induction.

328 Bistable systems usually exhibit hysteresis. For this reason, we expected that at pH
329 7.4 where both the 'ON' and 'OFF' states are attainable, past cell history would
330 influence competence induction. From our previous experiments we determined that
331 cells coming from acid precultures inoculated at pH 7.4 do not develop competence at
332 any density of inoculation (Figure 5b left panel, second column). We then studied
333 whether there is history-dependence by inoculating cells coming from non-acid
334 precultures at pH 7.4. We found that cells coming from a non-acid preculture became
335 competent at densities above OD_{595nm} 2.4 x 10^{-4}, which demonstrates that cell history
336 can influence competence development (Figure 6c). Past history has an effect on
337 competence because it determines the state of the machinery for CSP production,
338 which is 'OFF' when cells come from acid preculture but 'ON' when they come from
339 non-acid conditions. This explains why the effect of non-acid cell history appears from
340 a minimum inoculation density, since enough cells need to be inoculated in order for
341 them to produce the amount of CSP necessary for the system to remain 'ON' (Figure

342 6a inset). We then hypothesized that at pH 7.3 the critical inoculation density of cells
343 coming from non-acid history would have to be even higher than the one at pH 7.4 as
344 the model predicted that higher initial concentration of CSP would be necessary for the
345 system to remain 'ON' at lower pH. We confirmed this prediction experimentally by
346 showing that at pH 7.3 competence does not develop for an inoculation density of
347 OD_{595nm} 2.4 x 10^{-4} (as for pH 7.4) but from 7.4 x 10^{-4} upwards (Figure S6). Thus, our
348 results show that, as a consequence of the positive feedback involved in CSP
349 production, past cell history can determine whether competence is induced or not by
350 modifying the state of the machinery for CSP production and/or sensing.

351

352 **Discussion**

353 Recently, the view that bacteria use autoinducers as QS signals has been debated since
354 autoinducer concentration can change in response to the environment. Here, we show
355 experimentally that cell density, pH and antibiotic stress simultaneously regulate
356 competence development in *S. pneumoniae,* a system classically framed in the
357 paradigm of QS (Figures 2-5). Using a mathematical model we show that this occurs
358 because pH and antibiotics modify the rates at which single cells produce and sense
359 CSP and therefore the strength of the positive feedback loop coupling CSP detection to
360 CSP production (Figures 4 and 5). Importantly, this environmental dependency does
361 not override regulation by cell density but rather modulates the relationship between
362 the number of cells and the CSP concentration. Furthermore, we show that cell history
363 can also regulate competence development by modifying the status of the machinery to
364 produce and respond to CSP (Figure 6). Hysteresis in the competence response might

365 be particularly important in the natural niche of the pneumococcus, the human
366 nasopharynx. In particular it is consistent with the observation that there is constitutive
367 upregulation of competence in pneumococcal biofilms during nasopharyngeal
368 colonization[36]. In this context, once competence is triggered for the first time cells
369 would be primed to rapidly initiate another round of competence.

370 Why is competence controlled by CSP? Our results provide evidence against the
371 hypothesis that CSP is a timing device and in particular against the view that
372 competence can develop in a cell-density independent manner[18,19,22] (Figures 2 and 3).
373 Regarding the hypothesis that CSP is a probe to test diffusion [23], our findings suggest
374 that focusing on diffusion alone oversimplifies the information and functionality that
375 cells can gather through CSP production. We hypothesize that by releasing and
376 responding to CSP, bacteria can coordinate the development of competence and in
377 particular the expression of fratricins and bacteriocins, which are under the control of
378 the competent state. These proteins can lyse or inhibit the growth of surrounding cells
379 that are not competent, increasing the efficiency of genetic transformation and
380 mediating competition with other bacteria [37–40]. By coordinating competence
381 expression via CSP, an isogenic bacterial population can increase the total
382 concentration of secreted fratricins and bacteriocins, which likely translates into a
383 higher amount of lysed cells and therefore potential DNA donors. Importantly,
384 coordinating competence expression can also prevent the killing of clonal siblings
385 since immunity to these proteins comes with the competent state.

386 What is the relevance of the information carried by CSP? As shown by our model,
387 alkaline pH and antibiotic stress induce competence by increasing the rate at which
388 single cells produce and sense CSP. We expect this to be a general mechanism by

389 which sources of stress that are alleviated through competence induce this state (e.g.
390 mobile genetic elements as hypothesized by Croucher et al.[41]). Upregulating
391 competence in the presence of antibiotics can increase survival by activating the
392 expression of stress response genes [10,21], facilitating repair of damaged DNA and
393 mediating acquisition of resistance [21,42]. Our findings suggest that strategies to prevent
394 competence development in response to antibiotics can focus on counteracting the
395 effect of antibiotics on the rate at which cells produce or sense CSP. Regarding the
396 benefits of upregulating competence with alkaline pH, these are less clear and could be
397 an example of a non-adaptive response resulting from the inherent biochemical
398 properties of ComAB and possibly ComD.

399 **General insights on QS**

400 Our findings support the view that functional hypotheses stressing individual factors
401 like diffusion or population density underplay the complexity of information integrated
402 by QS systems [7,43–47]. Although the term 'quorum sensing' overemphasizes the role of
403 population density, we advocate for keeping it due to its widespread use and the fact
404 that density modifies autoinducer concentration in all autoinducer production systems.
405 Crucially, QS should be used in a broad sense that acknowledges that bacteria integrate
406 other factors in addition to population density into their QS responses. This view might
407 be very useful for other autoinducer production systems like competence in *Vibrio*
408 *cholerae*, where the synthesis of the autoinducer, CAI-1, depends on the intracellular
409 levels of cAMP-CRP and therefore might incorporate information on the metabolic
410 status of the cell [48,49]. Also in other systems, clear links between signal production,
411 quorum threshold and environmental conditions have been shown to affect QS [50–54].

412 Given that many biotic and abiotic factors can modify autoinducer concentrations

413 [55], future work should aim to study the relevance of such factors in the natural context
414 where bacteria secrete autoinducers. Such work is crucial to assess whether
415 upregulating QS in response to a particular factor provides a benefit for bacteria or is
416 merely a result of the biochemical properties of the QS regulatory network. An
417 interesting possibility is that, as in other biological systems [56], bacteria could perform
418 collective sensing of the environment through social interactions. In this context, by
419 secreting autoinducers cells could share individual estimates of environmental
420 conditions (e.g. antibiotic stress) for which upregulating QS is beneficial. Then,
421 autoinducer secretion would provide a way to get a more reliable estimate of the
422 environmental conditions by allowing a population to pool estimates made by
423 individual cells. Importantly, such a role for autoinducer secretion would explain the
424 dependency of QS on both cell density and the environment.

425

426 **Methods**

427 **Bacterial strains and growth conditions**

428 All pneumococcal strains used in this study are derivatives of the clinical isolate *S.*
429 *pneumoniae* D39 [28]. In order to monitor competence development all strains contain a
430 transcriptional fusion of the firefly *luc* and the *gfp* gene with the late competence gene
431 *ssbB*. See Table S1 for a list of the strains used and the Supplemental information for
432 details on the construction of the strains. *S. pneumoniae* was always grown in C+Y
433 complex medium [27] at 37ºC.

434

435 **Density and luminescence assays**

436 Cells were pre-cultured either in acid C+Y (pH 6.8) or in non-acid C+Y (pH 7.9) at
437 37ºC to an OD_{595nm} of 0.1. Right before inoculation, they were collected by
438 centrifugation (8000 rpm for 3 minutes) and resuspended in fresh C+Y (pH 7.9).
439 Luciferase assays were performed in 96-wells plates with a Tecan Infinite 200 PRO
440 Illuminometer at 37ºC as described before [57]. Luciferin was added at a concentration of
441 0.5 mg/mL to monitor competence by means of luciferase activity. Optical density
442 (OD_{595nm}) and luminescence (relative luminescence units [RLU]) were measured every
443 10 minutes. The time and density of competence initiation correspond to the first time
444 point where the RLU signal is equal or above 200 units. RLU is used instead of
445 RLU/OD because 1) when competence develops the rate at which the RLU signal
446 increases is faster than the growth rate and 2) due to the very low inoculation densities
447 used for Figure 2 the RLU/OD can be very high at the start (clearly before competence
448 has developed). The value of 200 units was chosen because once this value is reached
449 competence always developed. The effect of pH on competence development was
450 studied by inoculating cells in C+Y at a range of pH values from 6.8 to 9. pH was
451 adjusted by adding HCl and NaOH. The effect of antibiotics was studied by adding
452 streptomycin (3 $\mu g\ mL^{-1}$) and HPUra (0.075 $\mu g\ mL^{-1}$) to C+Y.

453

454 **Time-lapse fluorescence microscopy**

455 Phase contrast and GFP images were obtained using a Deltavision Elite microscope
456 (GE Healthcare, USA) with Trulight illumination for the GFP signal at 32% intensity
457 output (filtered with a neutral density filter) and an exposure time of 0.3 sec. Time-
458 lapse videos were recorded by taking images every 10 minutes. The polyacrylamide

459 gel used as semi-solid growth surface was prepared with C+Y (pH 7.9) and 10%
460 acrylamide. Cells were pre-cultured in acid C+Y (pH 6.8) and right before inoculation
461 on the slide they were resuspended in fresh C+Y (pH 7.9) as explained before.
462 Microscopy images were analyzed using MicrobeTracker [58].

463

464 **Mathematical model**

465 A mathematical model of the network of competence regulation (Figure 1) was
466 developed as a system of ODEs. The model incorporates the protein interactions
467 involved in sensing CSP via the two-component system formed by ComD and ComE
468 and exporting it via ComC and ComAB. Additionally, it explicitly models the
469 interaction of ComE and ComE~P with the gene promoters of *comAB*, *comCDE* and
470 *comX*. This is important since ComE~P binds these promoters as a dimer introducing
471 non-linearity into the system, which underlies the observed bistability. Population
472 growth is logistic and it is assumed that all the cells are homogeneous. See the
473 Supplemental information for the equations and further description.

474

475 **Author Contributions**

476 SMG, RAS, MK, GSvD, and JWV designed research; SMG, RAS and MK performed
477 experiments; SMG and GSvD developed the model; SM, RAS, MK, FJW, GSvD, and
478 JWV analyzed data; and SMG, FJW, GSvD and JWV wrote the paper.

479

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488

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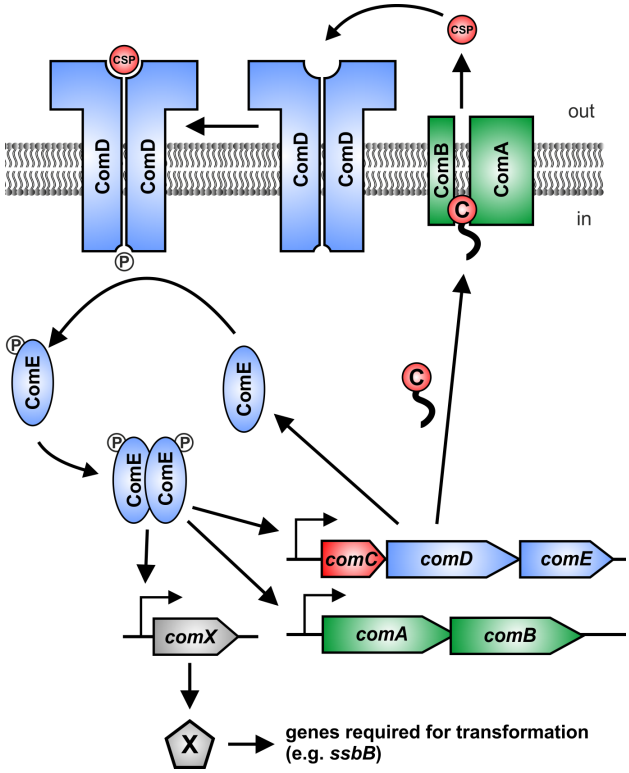
656

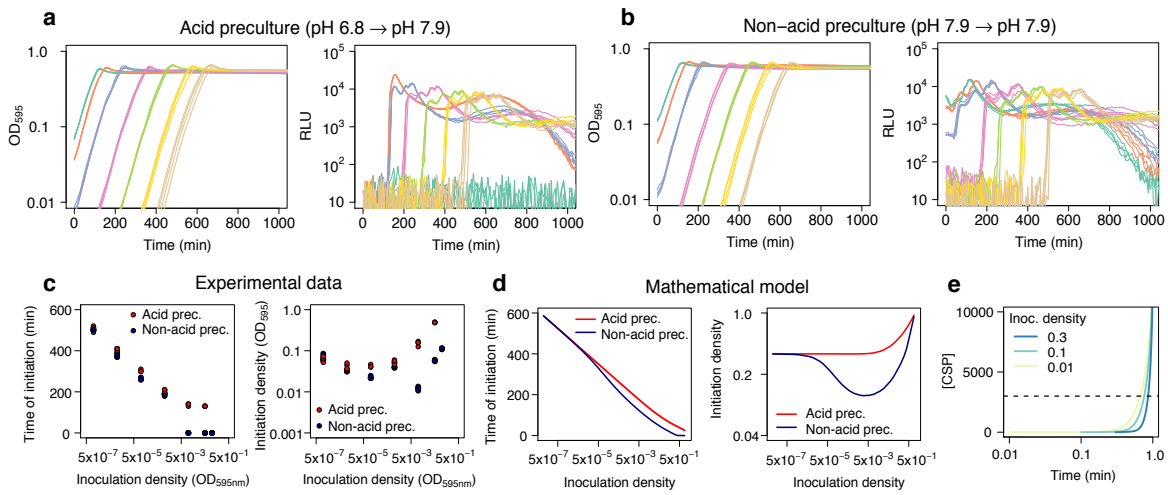
657 **Figures and Legends**

658



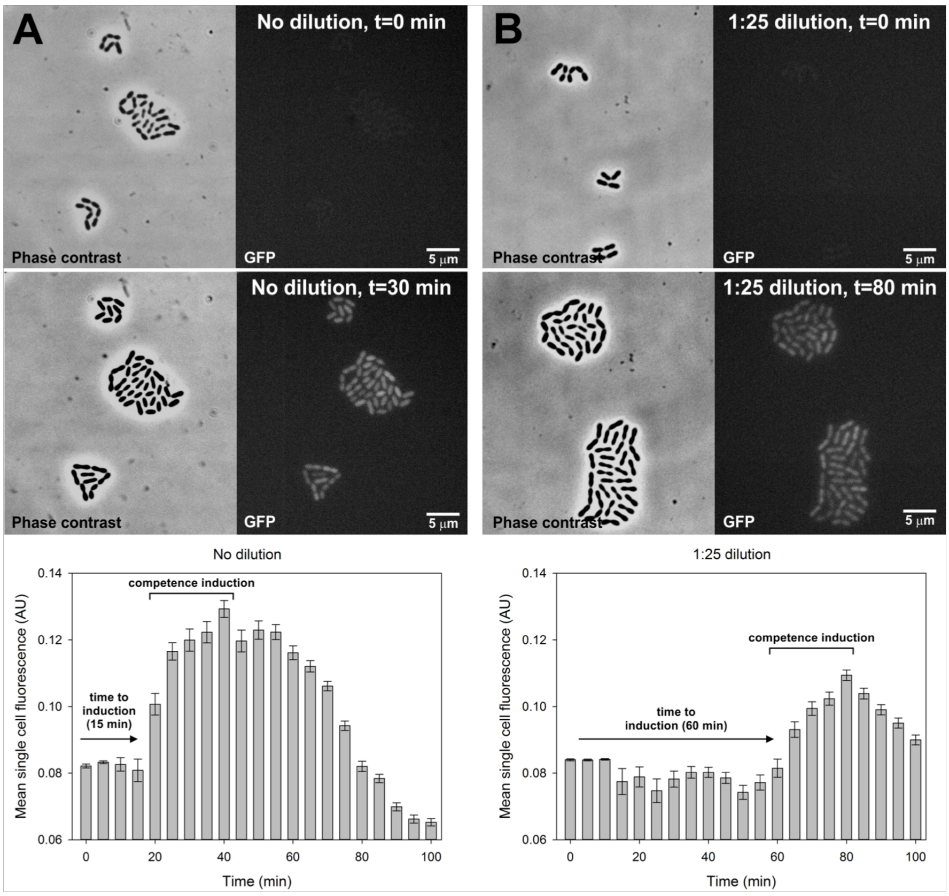
659

660 **Figure 1. Network of competence regulation in *S. pneumoniae*.** ComC (C) binds the
661 membrane protein complex ComAB, and it is processed and exported as CSP to the
662 extracellular space. CSP binds to the histidine kinase ComD, which is located in the
663 membrane as a dimer. Upon CSP binding, ComD autophosphorylates and transfers the
664 phosphate group to the response regulator ComE [12,26]. The phosphorylated form of
665 ComE (ComE~P) dimerizes and activates transcription of *comAB, comCDE* and *comX*
666 by binding to their promoters [11,12]. Unphosphorylated ComE can also bind these
667 promoters, repressing their transcription [26,59]. Synthesis of the alternative sigma factor
668 ComX directs transcription of genes required for genetic transformation as well as
669 other functions [10,27]. Two key features of this network are the presence of a positive
670 feedback loop (since increasing CSP detection leads to increasing CSP production) and
671 of non-linearity (since ComE~P interacts with the gene promoters as a dimer).



672

673 **Figure 2. Competence is regulated by cell density.** (**a,b**) Growth curves (OD_{595nm})
674 and competence expression measured as relative luminescence units (RLU) expressed
675 from the promoter of the late competence gene *ssbB* for populations inoculated at a
676 range of densities and grown at pH 7.9 in C+Y medium. In **a**, cells were precultured
677 under acid conditions (pH 6.8), while cells were precultured under non-acid conditions
678 (pH 7.9) in **b**. Four replicates are shown for each of seven inoculation densities
679 (OD_{595nm}): 0.1 (Green), 0.05 (Red), 0.01 (Blue), 10^{-3} (Purple), 10^{-4} (Light green), 10^{-5}
680 (Yellow) and 10^{-6} (Brown). Competence does not develop in cells coming from acid
681 preculture and inoculated at a density of 0.1. **c**) Effect of inoculation density on the
682 time until competence initiation (left panel) and the population density at which
683 competence was initiated (right panel). Competence initiation was defined as the time
684 where the RLU signal exceeded 200 units. **d**) Predictions of the mathematical model
685 concerning the effect of inoculation density on the timing of competence initiation (left
686 panel) and the density at which competence initiates (right panel). In the model,
687 competence initiation was defined as the time where the total concentration of ComX
688 times the population density exceeds 2000 units. Non-acid preculture is simulated in
689 the model by setting the initial amount of all proteins in the competence regulatory
690 network to the value they attain when cells are competent. **e**) The model predicts that
691 populations inoculated at lower densities will reach a threshold CSP concentration
692 (dotted line) at a lower density than populations inoculated at higher densities. The
693 effect of inoculation density on the time of competence development is also observed
694 in an unencapsulated mutant (Figure S2).



695

696 **Figure 3. The effect of initial cell density on the timing of competence**
697 **development is also observed by time-lapse fluorescence microscopy.** Phase
698 contrast and GFP images of cultures started at two different inoculation densities. In **a**
699 the slide containing C+Y medium at pH 7.8 was inoculated with 1.5 µL of cells pre-
700 cultured to OD_{595} 0.1 at pH 6.8 whereas in **b** it was inoculated with 1.5 µL of a 1:25
701 dilution of the same preculture. The first images (t=0) were taken right after
702 inoculation and the second ones correspond to the time point where the GFP signal per
703 cell is maximum. Image analysis shows that competence starts after 15 min in cells
704 directly inoculated from the pre-culture, whereas it takes 60 min for cells coming from
705 the 1:25 dilution of the preculture. A typical outcome of such an experiment is shown.
706 The error bars correspond to the standard error of the mean.

707

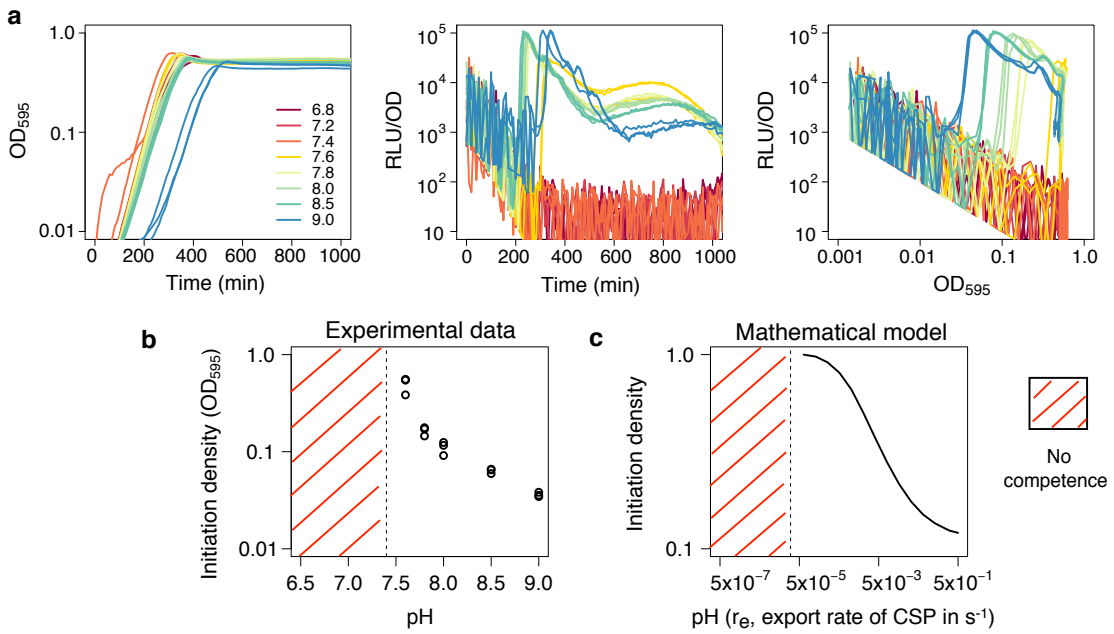


Figure 4. **Competence is upregulated by higher pH. a**) Effect of pH on growth curves (left panel) and the dynamics of competence expression (middle panel). Competence expression was quantified as relative luminescence units (RLU) normalized by cell density. In the right panel, competence expression is plotted in relation to cell density. All populations were grown at the indicated pH and inoculated at a density of OD_{595nm} 0.002. Three replicates are shown for each pH. **b**) Effect of pH on the population density at which competence was initiated (the density at which RLU exceeded 200 units). Competence did not develop at pH 7.4 and below. **c**) Predictions of the model on the effect of the rate of CSP export, r_e, and thus the pH, on the density of competence initiation. Competence does not develop anymore below a threshold rate of CSP export.

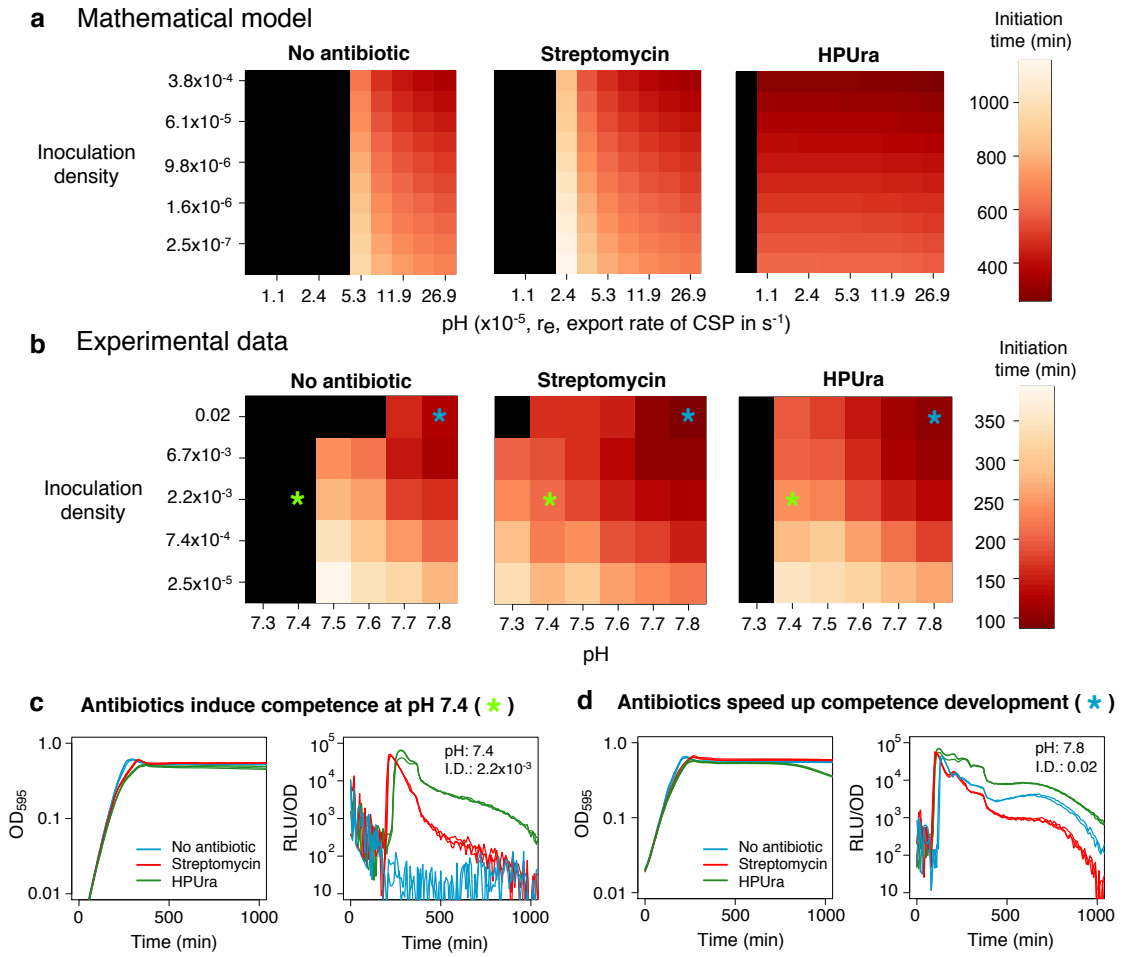


Figure 5. Competence is simultaneously regulated by cell density, pH and antibiotic stress. Predictions of the mathematical model (**a**) and experimental data (**b**) on the dependency of the time of competence initiation on inoculation density, pH and antibiotic stress. The x-axis in **a** corresponds to the rate of CSP export in the model, r_e, which is a proxy for pH. The color scales with the time of competence initiation with more intense red corresponding to faster development of competence. Black represents no competence development. In **b** each box corresponds to the average initiation time of three replicates. Both the model and the experimental data show that competence develops faster at higher pH and higher inoculation densities. **c**) Antibiotics induce competence at pH values that repress natural competence development. **d**) At pH values that are not repressive for competence development (pH > 7.4), competence develops faster in the presence of antibiotics. The stars indicate which conditions are plotted in **c** and **d**.

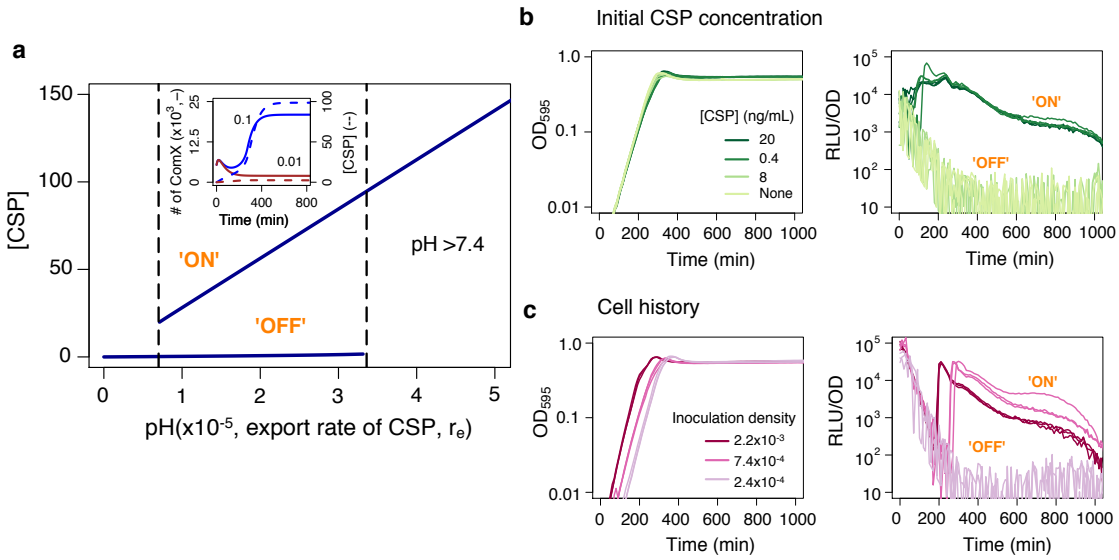


Figure 6. A bistable regime for competence development. a) Extracellular concentration of CSP in response to the rate of CSP export. The model predicts the existence of a region where competence always switches on regardless of the initial conditions (which would correspond to pH > 7.4) and of a bistable 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).