Peptide signaling without feedback in signal production operates as a true quorum sensing communication system in *Bacillus subtilis*

Iztok Dogsa¹,³, Mihael Spacapan¹, Anna Dragoš³,², Tjaša Danevčič³, Žiga Pandur¹ & Ines Mandic-Mulec¹,³

Bacterial quorum sensing (QS) is based on signal molecules (SM), which increase in concentration with cell density. At critical SM concentration, a variety of adaptive genes sharply change their expression from basic level to maximum level. In general, this sharp transition, a hallmark of true QS, requires an SM dependent positive feedback loop, where SM enhances its own production. Some communication systems, like the peptide SM-based ComQXPA communication system of *Bacillus subtilis*, do not have this feedback loop and we do not understand how and if the sharp transition in gene expression is achieved. Based on experiments and mathematical modeling, we observed that the SM peptide ComX encodes the information about cell density, specific cell growth rate, and even oxygen concentration, which ensure power-law increase in SM production. This enables together with the cooperative response to SM (ComX) a sharp transition in gene expression level and this without the SM dependent feedback loop. Due to its ultra-sensitive nature, the ComQXPA can operate at SM concentrations that are 100–1000 times lower than typically found in other QS systems, thereby substantially reducing the total metabolic cost of otherwise expensive ComX peptide.
Bacteria secrete and share quorum-sensing (QS) signal molecules (SM) that bind to specific receptors and induce cell density-dependent adaptive responses\(^1,2\) and affect microbial community interactions when the critical concentration of SM is reached\(^3\). Not every bacterial communication system is QS (Fig. 1a)—only the communication systems where bacterial response follows a sharp transition dynamics from basic to the maximum response level is true quorum sensing\(^5\). To achieve this, most QS systems incorporate the coupling between signal production and signal detection with the signal amplifying its own product, although maximum response level can be reached also when signal auto-amplification is artificially broken\(^6\). In contrast, the dynamics of signal molecule synthesis and response in communication\(^7,8\) systems without positive feedback loop regulation has been little studied so far, especially, quantitative research on encoder (signal molecule production) and decoder modules (response to signal molecule) comprising communication system is missing\(^9\). We close this knowledge gap by studying the ComQXPA communication system of *Bacillus subtilis*, where signal molecule production is not coupled to signal molecule detection\(^10-15\).

In general, quorum sensing encoder module that encodes the information about the cell density into the signal molecule (SM) concentration can be classified according to its sensitivity to cell density. When SM concentration increases faster than linearly with cell concentration, one can call such encoder module ultra-sensitive\(^9\). The classification of decoder modules seems to be less complete, therefore, we follow the general definition of ultra-sensitivity in molecular biology: ultra-sensitivity describes an output response that is more sensitive to stimulus change than the hyperbolic Michaelis–Menten response\(^14\). In the communication systems the stimulus is signal molecule (SM) and response (RM) is the expression level of gene dependent on SM. The ultra-sensitive communication system has ultra-sensitive encoder and decoder module.

The ComQXPA communication system is likely not unique to *B. subtilis* species and its close relatives, as comQXPA-like loci are predicted to occur outside the *B. subtilis* clade, including some species of *Clostridiales* order\(^15\). No known regulators of comQXPA operon expression exist, it is however known that the operon is not expressed in the presence of superoxide radicals\(^16\). The ComQXPA communication system (Fig. 1b) involves the ComQ isoprenyl-transferase, which is required for the maturation of the ComX signal peptide. The mature ComX, which is a signal molecule (SM) of interest in this manuscript, is first synthesized as a 55-residue propeptide and then processed and modified by ComQ\(^11,17\). Depending on the strain-specific type (phenotype)\(^18\), the mature ComX exists as isoprenylated 5–10 amino-acid peptide\(^19\) that once secreted can bind to the membrane receptor histidine kinase ComP, which triggers phospho-transfer to ComA\(^17,18,20\). Phosphorylated ComA directly modulates the expression level of various genes, including the expression level of *srfA* operon\(^12\), which serves as a measure for the response (RM) to the signal molecule SM (Fig. 1b). Although the *srfA* expression required for the synthesis of the major lipopeptide antibiotic surfactin\(^20\) also depends on other extracellular peptide signaling systems from the Rap-Phr family\(^21,22\), the research of this paper is focused on ComX dependent response. For true quorum sensing, which is regarded as a population density-driven event, one would expect that most of the cells will be involved in the communication. Ideally, every cell produces signaling molecules, shares signaling molecules, and coordinately responds to the signaling molecules. In recent years, however, it was shown that the expression of signaling molecules can be heterogeneous\(^23-26\).

When the concentration of signaling molecule reaches a threshold value, a coordinated and homogeneous expression of target genes may be initiated in all cells of the population\(^5,27\) or a heterogeneous gene expression in the population may be triggered at low concentrations\(^25,27,28\). However, these studies were performed on communication systems with positive feedback loop regulation, where signal molecule (SM) enhances its own production. How heterogeneous is the population of signal producers and responders in communication systems lacking feedback loop regulation, like the ComQXPA in *B. subtilis*, is to the best of our knowledge, unknown.

It was theoretically estimated that among SM, peptide signals of Gram-positive bacteria are more than 20 times metabolically more expensive than AHLs produced by Gram-negative bacteria\(^29\). The existence of fitness cost of signal molecule production in Gram-negative bacterial models has been theoretically predicted\(^30-32\) and experimentally supported\(^33\). One would thus expect that the fitness burden of metabolically costly SM production in Gram-positives is even more pronounced.

In order to determine the operational mode of the communication system without the SM dependent feedback loop, we quantified the system’s core parameters dynamics by modeling...
and experimental approach. In particular, we provide data on the time-dependent dynamics of (i) the concentration of the signal molecule SM (i.e., ComX), (ii) the cell density \(N\), (iii) the critical SM concentration to elicit minimal quantifiable response (lower limit of response-LLR), (iv) the response (RM) to the SM, represented by the expression of the srfA operon encoding the surfactin synthetase, (v) the population distribution of signal molecule producers and signal responders.

Our results show that the ComQXPA communication system functions as a true QS system that adopts a switch-like dynamics, which is achieved by linking ultra-sensitive encoder module (signal production) with ultra-sensitive response module (signal response). The non-linear increase in signal molecule synthesis is coupled to the growth rate and oxygen concentration. We also show that signal production and response are predominantly spread over the entire population with limited heterogeneity. A very low concentration of costly signal molecule is sufficient for triggering the QS response, which can explain the observed low metabolic cost in signal molecule production.

**Results**

The concentration of signal molecule ComX increases by the square of bacterial density. In order to determine the dynamics of signal molecule (ComX) production, we have used experimental and mathematical modeling approaches. We quantified the ComX concentration over time in the spent medium of PS-216 (\(\Delta\text{comP}\)) with the biosensor strain BD2876 (for strain description see Supplementary Table 1), which produces β-galactosidase in response to the exogenous addition of ComX \(^{21}\). The assay included proper controls and calibrations to assure the biosensor-derived ComX concentrations are accurate (for details see Materials and methods). We found that the ComX concentration correlated positively with population density of PS-216 (\(\Delta\text{comP}\)) and remained constant at 10 nM after entering the stationary phase (Fig. 2a). Importantly, the representation of ComX concentration versus cell density (OD650) (Fig. 2b) showed a non-linear trend between the two parameters. The experimental data were fitted by an allometric function:

\[
SM(t) =aN(t)^b
\]

Where \(SM(t)\) is a signal molecule (ComX) concentration in time, \(N(t)\) is bacterial cell density in time, expressed as optical density of the bacterial suspension (OD650). The fitting results for parameters \(a\) and \(b\) were (9.6 ± 0.6) nM a.u. \(^{-2.09}\) and 2.09 ± 0.10, respectively. The value of parameter \(a\) means that at OD650 = 1.0 a.u., which corresponds to the stationary growth phase in our experimental conditions and the bacterial density of \(4 \times 10^8\) cells mL\(^{-1}\), the ComX concentration is about 10 nM. In the early exponential growth phase the concentration was about 0.1 nM. Considering parameter \(b\), the value obtained (2.09 ± 0.10) indicates that the ComX concentration increases by the square of bacterial density. This means that with increasing population density, the SM concentration (ComX) increases by the second power, while the amount of ComX per cell increases linearly. This relationship suggests that ComQXPA has an ultra-sensitive encoder module \(^{22}\), where signal molecule production is very sensitive to cell density. The same mathematical relationship can be obtained by assuming that SM production rate per cell corresponds to the product of a specific cell growth rate and a cell density (i.e., population growth rate, for details, see Supplementary Methods, Derivation of ComQXPA communication system model). The dependence of the SM production rate per cell on (a) cell density and (b) the specific cell growth rate can be seen as an alternative way to obtain the ultra-sensitivity of encoders, which is usually achieved by SM dependent positive feedback in many QS systems \(^9\). This makes ComX a true indicator of population density, which also encodes information about the cell growth rate.

The production of signal molecule ComX in native concentration does not present a substantial metabolic burden for the producer. Peptide signal molecules (SM) used by Gram-positive bacteria are metabolically costly to produce \(^{22}\). We estimate here that single molecule of ComX produced by \(B.\ subtilis\) used in this study (pherotype 168) requires a considerable investment of 484 ATP units per single signal molecule (for calculation details see Supplementary Methods, Calculation of ATP requirements for synthesis of 1 SM, ComX, 168 pherotype). This drastically exceeds the estimated cost of typical Gram-negative bacterial QS signals, with butyryl-homoserine lactone, C4-HSL from *Pseudomonas aeruginosa* costing only 8 ATP units \(^{22}\). However, the concentration of stationary growth phase signaling peptide ComX is 100–1000 times lower in *B. subtilis* (10 nM, Fig. 2a) compared to the typical concentrations of AHLs released by Gram-negative bacteria \(^{35–38}\). This suggests that high cost per SM is buffered by low concentrations of SM, thereby reducing the fitness costs of SM production in peptide-based communication systems. In order to test the metabolic cost of ComX production, we first compared the growth curves of receptor-deficient PS-216 (\(\Delta\text{comP}\)) and signal and receptor-deficient PS-216 (\(\Delta\text{comQXP}\)) strains (Fig. 2c). The use of the strains without a receptor made it possible to separate the costs of signaling from the additional costs of the communication response.

![Figure 2a: ComX concentration over time in the spent medium of PS-216 (\(\Delta\text{comP}\))](https://example.com/fig2a)

![Figure 2c: Growth curves of PS-216 (\(\Delta\text{comP}\)) and PS-216 (\(\Delta\text{comQXP}\))](https://example.com/fig2c)

Apparent, the maxima of growth curves of \(\Delta\text{comP}\) and \(\Delta\text{comQXP}\), and their slopes (corresponding to the growth rate divided by log 2) were almost identical: PS-216 \(\Delta\text{comP}\) = (0.503 ± 0.008) h\(^{-1}\) and PS-216 \(\Delta\text{comQXP}\) = (0.496 ± 0.007) h\(^{-1}\), suggesting that ComX production does not represent a substantial metabolic burden in the observed system (Fig. 2c). The more direct fitness comparison between PS-216 (\(\Delta\text{comQXP}\)) and (\(\Delta\text{comP}\)), was carried out through a competition assay between PS-216 (\(\Delta\text{comQXP}\)) and PS-216 (\(\Delta\text{comP}\)) (Fig. 2d). In line with results in Fig. 2c, ratio of ComX producers and ComX non-producers did not change considerably throughout the experiment, suggesting negligible costs for signal production (Fig. 2d).

Next, we tested whether the absence of prudent SM production induces measurable fitness cost. To test this, we overexpressed *comX* from the *P. pyocina* IPTG-inducible promoter (Supplementary Fig. 2a, b), which ensured the production of additional copies of ComX. As expected, the overproduction of ComX has a negative impact on the growth of *B. subtilis* (Supplementary Fig. 2a). The overexpression of ComX in *E. coli* had a similar negative fitness effect (Supplementary Fig. 2c). As it can be calculated from Supplementary Fig. 6b, the concentration of ComX in *E. coli* spent media was about 900 nM, corresponding to 200 nM a.u. \(^{-1}\), which is about 20 times more than we have measured in *B. subtilis* (Fig. 1a). The above results indicate that the costs of ComX synthesis under the native production regime are very low and can only be evaluated under non-native overexpressing conditions.

The ComQXPA communication system operates in strong correlation with the oxygen concentration. As already mentioned, the SM production rate per cell in ComQXPA is not controlled by the SM dependent positive feedback loop, but by cell density and specific cell growth rate (population growth rate), (eq S5–S6). The question is how bacteria then sense cell density and specific growth rate, which accelerate signal production. One of the key factors for the growth rate of *B. subtilis* is...
n = in co-culture; each time OD650 reached 0.6 a.u. the co-culture was transferred to the fresh medium; performed and each time 6 of 7 transfers were checked for CFUs of both strains.

The slopes do not differ significantly (P = 0.32; ΔcomP = (0.496 ± 0.007) h⁻¹ and ΔcomQXP = (0.503 ± 0.008) h⁻¹). The same strains grown in co-culture; each time OD650 reached 0.6 a.u. the co-culture was transferred to the fresh medium; n = 3 biologically independent experiments were performed and each time 6 of 7 transfers were checked for CFUs of both strains.

First, we did not allow any aeration of the batch culture, i.e., the oxygen supply to the growing culture was limited by diffusion of air through air filters on the inlets of the incubator. We monitored changes in oxygen concentration during growth in batch culture and observed an almost perfect negative correlation between the growth curve and the dissolved oxygen concentration (Fig. 3a, Supplementary Fig. 3). The strongest decrease in oxygen content occurs at the point where the population growth rate reached its maximum. When spent medium of PS-216 wt was tested by the ComX biosensor BD2876 (ΔcomQ, srfA-lacZ), we could measure the significant response (for t > 1.75 h, P < 0.008) by the biosensor that increased with the growth of the culture (Fig. 3c), indicating ComX is being produced. This agrees with Fig. 2a, where we quantified the produced ComX in the spent medium of PS-216 (ΔcomP, producing ComX, but not responding to ComX). As expected for the proper ComX biosensor, it barely responded to the tested spent medium with no ComX (PS-216 ΔcomQ spent medium) and strongly responded to the same medium when purified ComX was added (Supplementary Fig. 4) confirming the ComX is the major factor being measured by the biosensor BD2876.

Next, we assessed the signal production in batch culture, where we assured continuous oxygen saturation. Under this condition the negative relationship between population size and dissolved oxygen concentration is broken. Surprisingly, oxygen saturation eliminated ComX production, which can be seen by very low biosensor BD2876 response that is indistinguishable from the spent medium with no ComX (PS-216 ΔcomQ spent medium) even in the late-stationary growth phase (Fig. 3d). This result indicates that the ComQXPA communication system has lost its functionality, when there is no ‘natural’ oxygen gradient, and that the oxygen content can be used as an indicator of cell density and growth rate.

The response model shows that the response of the cells to ComX is non-linear. In our model, the expression level of the srfA operon serves as a measure for the response (RM) to the signal molecule SM, represented by ComX. To study how RM depends on SM we evaluated promoter activity of srfA in the B. subtilis PS-216 (ΔcomQ, P_srfA::yfp), which carries the markerless deletion of comQ and is therefore signal-deficient. Response level was assessed by incubating the PS-216 (ΔcomQ, P_srfA::yfp)
for 4 h in the presence of different ComX concentrations, which was the only factor that varied in this experiment. The response level was expressed as Yfp fluorescence per cell, normalized to the maximum response, $W_{\text{max}}$, which gives a relative measure, $W$, (SM), of how strongly the cells respond to ComX and this is shown as a function of the exogenously added ComX in Fig. 4a. The response to SM was sigmoidal. In order to check whether the response curve had reached the final shape after 4 h of biosensor incubation, we performed the same experiment, but incubated biosensor with ComX for 3 or 6 h, respectively. As can be seen from the comparison of Fig. 4a with Supplementary Fig. 5, the response curve in Fig. 4a with Supplementary Fig. 5, the response curve has not changed after extending the incubation over 4 h. We have therefore taken the 4 hours response curve as a function of the exogenously added ComX in Fig. 4a. The sigmoidal functions can typically describe the relationship between transcription factors and promoter activities, and can be modeled by the Hill equation\(^43,44\). In the case of ComQXPA the ComX dependent transcription factor ComA-P acts directly on the $P_{\text{Psrf}}\text{A}$ promoter and induces its activity as the response to the signal. Assuming a linear relationship between ComX concentration (SM) and the active ComA-P one can expect that the experimental data can be fitted by the Hill equation:

$$W(SM) = \frac{W_{\text{max}} SM^n}{Km^n + SM^n}$$  \hspace{1cm} (2)

$SM$ is ComX concentration and $Km$ is the ComX concentration at which half of the maximum response is achieved; $n$ (Hill coefficient) describes the cooperativity among transcriptional activators. Successful fits indicated by the low reduced $\chi^2$ (see Supplementary Table 2), show that the biosensor sensitivity is maximized at 3–5 nM of ComX ($Km$), while the highest response value is reached at around 10 nM of the ComX. $n > 1$ values obtained for all fits indicate positive cooperativity (i.e., ultrasensitivity\(^14\)) in the binding of the transcriptional factor ComA to the $srfA$ promoter\(^43,44\). This agrees with the research showing that two molecules of the ComA homodimer cooperatively bind to the two promoter regions located upstream of the RNAP binding sites of $srfA$\(^\text{13,20,45}\). The inactivation of the second promoter region decreases the promoter activity of $srfA$ by 100-fold (ref.\(^\text{13}\)), which underscores the importance of the second binding region, explains $n \geq 2$ and the sharp increase in $srfA$ promoter activity with ComX concentration. In addition, we show here that the critical concentration of ComX required to induce quantifiable response (designated here as lower limit response (LLR)) is 0.2–0.5 nM. These results, therefore, suggest that the response per cell depends cooperatively on the ComX concentration and that the cells respond to very low concentrations of ComX.

Fully functional ComQXPA communication system does not require a positive feedback loop—the validation of the ComQXPA communication system. The response curve in Fig. 4a is a function of the SM concentration only. In the more natural setting (i.e., during growth) the cells encounter growth-dependent changes in SM concentrations as well as changes in bacterial density and growth rate over time. We have therefore...
asked whether the response curve based on the modeling and results presented in Figs. 2b and 4a could fit the response data in the SM producing and responding strain exposed to changes in these three parameters.

We cultivated the SM producing and responding PS-216 strain carrying the response reporter (PsrfA-lacZ) in a large volume bioreactor system (Fig. 4b). This allowed sterile sampling of spent medium and cells (for response quantification) at several time points, without affecting growth conditions. Immediately after the inoculation of the fresh medium by overnight culture the β-galactosidase activity of PS-216 (PsrfA-lacZ) was high. We assumed that this was a consequence of the accumulation of the expressed PsrfA reporter (β-galactosidase, RM) during the overnight growth. As a consequence of the dilution of the intracellular β-galactosidase (RM) due to cell division, the activity of the β-galactosidase decreased sharply after 2 h incubation (Fig. 4b). Simultaneously, as predicted by (Eq. 1), the concentration of SM (ComX) in the medium was increased exponentially during growth, and so reached a critical concentration to activate the srfA promoter. In particular, as elucidated by the fits of (Eq. 2) to the data in Fig. 4a, the lower limit of the response (LLR) is reached shortly before upturn of the cell response curve in Fig. 4b. At this point the culture is in exponential growth phase at the cell density of 3 to 8 × 10^7 cells mL^{-1}. The steep slope of the response curve indicates that the rate by which the response molecule (RM) is synthesized now exceeds the dilution due to the cell division rate. From now on, the response per cell correlates approximately linearly with OD650, suggesting a strong coupling to cell growth. Taking these facts into account and considering that the response molecule (RM) concentration is sensitive to the concentration of the signal, SM, (Eq. 2) and that SM can be expressed in terms of cell density (Eq. 1), the concentration of a response molecule per cell, RM(t)/N(t), can be analytically described (see also Supplementary Methods, Derivation of ComQXPA communication system model) as:

\[
\frac{RM(t)}{N(t)} = \frac{RM0}{N(0)} + \frac{RM1(t)}{N(t)}
\]

where RM0/N(0) is the response per cell of overnight culture, i.e., the overnight accumulated β-galactosidase. The second term, RM1(t)/N(t) accounts for the synthesis of the β-galactosidase after inoculation of a fresh medium and comprises the parameters describing the sensitivity of the response to a signal molecule, Wmax, Km, n (Eq. 2), the signal production, a (Eq. 1), cell density, N(t) and proportionality constant, k that gives the magnitude of the response per cell when the potential to respond to the signal is maximally fulfilled (i.e., at Wmax) and the specific growth rate is 1 h^{-1}. The definition of RM1(t) is given in Supplementary Methods (eq S11). Note that for the derivation of Eq. 3) we assumed no degradation of SM occurs, as our experiments suggest SM is stable under the conditions studied (Supplementary Figs. 6a and 7, see also Supplementary Methods, Derivation of ComQXPA communication). All the parameters in (Eq. 3), except k in RM1(t) and RM0, were taken as constants obtained in the independent experiments by fits of (Eq. 1) and (Eq. 2). With k and RM0, as the only fitting parameters, we applied the mathematical model in (Eq. 3) (for details of the model equation see Supplementary Methods, Derivation of ComQXPA communication system model) to fit the experimental cell response data (Fig. 4b). The successful fit (see Supplementary Table 4 for details) indicates that the relationship assumed among cell density, cell growth, signal concentration and response in (Eq. 3) is valid and yields (760 ± 120) M.U. for k and (5.5 ± 1.5) M.U. a.u. for RM0. Again, we did not need to incorporate the SM feedback loop into our model, which is consistent with published results suggesting that this communication system lacks a feedback loop^{10-13}.

The ComQXPA dependent signaling and response at the cellular level. So far, we have focused on the population averages, which is a traditional approach in studies on microbial communication systems^{17,46}. We here report results on communication dynamics of B. subtilis at the single cell level using fluorescence-based molecular tools. This approach provides the means to track temporal changes in expression of genes involved in signal synthesis (signaling) and in response and thus provides the insight into a phenotypic heterogeneity within the population.

We used the double-labeled fluorescent strain B. subtilis PS-216 (comQ-yfp, srfA-cfp), in which fluorescent reporters were
fused to the $comQ$ and $srfA$ promoters. The two genes code for the ComX signal-processing protein and the communication-activated operon, respectively. Since $comQ$ and $comX$ share the same promoter and their genetic sequences often overlap, $comQ$ expression level of $comQ$ corresponds to the expression level of $comX$. The fluorescence of individual cells was observed under the microscope in different growth phases and quantitatively analyzed (Fig. 5).

The observed expression pattern for the signaling gene ($comQ$-$yfp$) follows lognormal distribution (Fig. 5g). A small number of outliers in the $comQ$ expression (on average, 10x brighter than the majority) were easily detected in the qualitative image analysis (Fig. 5a). These hyperproducers were not present in the overnight culture and began to occur during exponential growth, after 1-hour incubation in fresh medium, (Fig. 5d). In general, hyperproducers accounted for about 0.1–1% of the population, and their frequency increased during the first 6 hours. These data suggest that bulk of the ComX is nevertheless produced by the majority of the population as expected for the true QS communication systems. The contribution of the $srfA$ hyperproducers to the total surfactin production is even less pronounced since their occurrence did not exceed 0.1% of the population (Fig. 5b, d).

The most heterogeneous expression of the communication signaling gene ($comQ$-$yfp$) was observed in overnight culture, immediately after its transfer to the fresh medium (Fig. 5g, Supplementary Fig. 8a), but hyperproducers where not detectable at this time (Fig. 5d). Once the cells began to divide, the distribution shifted to lower fluorescence intensities with a simultaneous decrease in heterogeneity, but from 3 to 4 h onwards single cell fluorescence gradually increased, along with an increase in population heterogeneity (Fig. 5e, g, Supplementary Fig. 8a). This suggests that the expression rate is now higher than the division rate (i.e., the production overpowers the dilution due to cell division). A similar pattern was observed in the communication response ($srfA$-$cfp$) (Fig. 5f, h, Supplementary Fig. 8b) with two major differences. The minimum level in $comQ$-$Yfp$ fluorescence was reached 1 h later than $srfA$-$Cfp$ fluorescence, which suggests the expression of ComX is in first hours low compared to the $srfA$ expression. Nevertheless, the entire cell population, with the exception of hyperproducers, which represented only a fraction of the $comQ/srfA$ expressing cells, followed unimodal lognormal distribution expression pattern. This suggests that the ComQXPA communication phenomenon in B. subtilis, at least under the conditions in our experiment, is not restricted to individuals and can be studied at the population level, i.e., the averages represent well the population.

The $comQ$-$yfp$ and $srfA$-$cfp$ expression co-localization analysis (Supplementary Table 5) reveals the correlation coefficient of about 0.5, which is significantly ($P = 0.01$) higher than in the overnight culture. The presence of the correlation suggests that on average, cells that produce the signal more intensively, also respond to signal more intensively, supporting the idea of self-sensing. However, the correlation coefficient strength was only moderate, suggesting that sensing of the external signal (sensing of others) still works as expected for a typical QS system.

**Discussion**

The variable peptide and isoprene moiety of ComX signaling molecule enables this molecule to be intra-species specific. High specificity has its price—our estimation of ComX molecule cost is 484 ATP, considerably more than 8 ATP for acyl-homoserine lactones (AHL) based communication systems. It was shown that cost of AHL synthesis is high enough to experimentally measure its fitness cost, which was for the peptide SM undetectable in our assays. The most obvious reason for this is that communication system in B. subtilis operates in nM concentration range (10 nM a.u.$^{-1}$, i.e., 10 nM of SM concentration per unit of culture optical density), whereas SM of other communication systems are often in μM concentrations.

This includes the study showing the measurable fitness cost of AHL, where AHL concentration was 20 μM a.u.$^{-1}$ (ref. 31). Therefore, our results imply that the problem of high cost per molecule of ComX peptide was mitigated by B. subtilis by deploying communication system that operates at very low concentrations.

The prerequisite for a well-functioning quorum sensing system is the ability of the signal molecule concentration to follow cell density, which is the role of encoder module. Drees et al. theoretically predict the ComX encoder module consisting of the $comQ$ and $comX$ gene, to be an Ideal class. In this class signal molecule concentration increases linearly with cell density, with the exponent b in Eq. (1) being 1, implying a constitutive synthesis of SM (see Supplementary Methods, Derivation of ComQXPA communication system model). However, our results indicate that SM (ComX) concentration in B. subtilis increases with the square of cell density, which classifies the encoder module as the Ideal ultra-sensitive ($b > 1$), meaning that the increase in signal molecule concentration is very sensitive to the increase in cell density and that SM production must be controlled by an additional factor. This is generally represented by a positive signal autoregulation, which is missing in ComX encoder module, which means that B. subtilis controls the production of SM (ComX) by sensing others (cell density) through an alternative mechanisms. According to our data this mechanism involves the detection of dissolved oxygen. First, B. subtilis produces ComX only in the oxygen diffusion limited medium, but not in an oxygen saturated medium, independent of cell density and growth rate. Second, the concentration of dissolved oxygen (DO) decreases sharply when the population growth rate increases and oxygen consumption exceeds supply (Supplementary Fig. 3). This occurs between 1.5 and 2.5 h after inoculation, which coincides with the increase in signal production and the LLR window, the earliest measurable

The induced response in ComQXPA communication system is graded and almost switch-like. The perfect QS system does not produce a response until the threshold bacterial density is reached and then immediately switches to a full response. This minimum to maximum transition may be either a perfect switch or a graded induction. By combining the information from Figs. 2b and 4a in the form of eq S9 results in the normalized ComQXPA response curve as a function of bacterial density (Fig. 6a) that resembles a graded switch like induction (compare to Fig. 1a). The perfect switch like communication system is unrealistic, because it requires that all the cells are perfectly synchronized and immediately switch to maximal response, leaving no time for the adaptation to the signal stimuli. It is reasonable to expect that for the true quorum sensing system (QS) most of the response has to occur within the same generation of dividing cells ($n_{gen} < 1$). As can be seen in Fig. 6b, this is true for the ComQXPA communication system (our case), which achieves 50% of the response within the same generation of dividing cells ($n_{gen} = 0.7$). On the other hand, if the communication system lacks the ultra-sensitivity in either the signal production (encoder module) or response production (decoder module), the achievement of 50% response shifts well over the same generation of dividing cells ($n_{gen} \approx 1.4$ to 1.6), extending the cell density and time needed for substantial response to occur.
Fig. 5 Single cell quantitative fluorescence microscopy of *B. subtilis* PS-216 (comQ-yfp, srfA-cfp). The fluorescence microscopy images were taken periodically during incubation of *B. subtilis* PS-216 (comQ-yfp, srfA-cfp) in a batch fermenter by the YFP filter (a), CFP filter (b) or DIC (c). The example YFP and CFP images, taken after 3 h of incubation were pseudo-colored. The scale bar represents 10 µm. *n* = 3 biologically independent experiments were performed. d % of population of cells that are hyper-expressing comQ-yfp or srfA-cfp is depicted. Gene expression level determined by single cell fluorescence microscopy was measured as Na-fluoresceinate standard normalized mean fluorescence intensity per cells expressing comQ-yfp (e) and srfA-cfp (f); ON is the overnight culture. One of the three qualitatively similar cell distributions is shown in (g) and (h) for comQ-yfp and srfA-cfp, respectively; areas under the curves are the same in all time points. For additional replicate see Supplementary Fig. 8.
response to the signal (Fig. 4b) followed by the upturn of the response curve. Our results thus underline oxygen as an independent factor influencing signal production and are in good agreement with experiments by Ghirbi et al.50, who observed a significant decrease in B. subtilis surfactin production, when DO was increased from 40 to 60%. The recent research reveals that surfactin promotes growth in early stationary phase by enhancing oxygen diffusion and that surfactin maintains viability upon oxygen depletion, which becomes critical at high cell densities41. This explains the factors influencing the production of ComX (SM in our study), a major signal for surfactin synthetase expression (RM in our study). Cells need surfactin, when their numbers expand quickly and oxygen is dropping. The mechanism on how oxygen modulates the ComX production remains to be elucidated, however, Ohsawa et al.16 showed that the expression of the comQXP locus decreases with high levels of superoxide (O$_2^-$). Since ROS production-rate is proportional to collision frequency of oxygen and redox enzymes, the rate of O$_2^-$ and H$_2$O$_2$ formation inside the cells depends directly on the oxygen concentration in the extracellular environment51–54. Although it might be tempting to conclude that in this case sensing superoxide is equivalent to sensing oxygen, it is important to note that the superoxide concentration is the “private” property of the cell, while oxygen concentration is shared by all cells and can carry the population-related information.

In general, a bacterium could achieve the same level of graded, nearly switch-like induction as in our case, by deploying the SM production with even higher level of ultra-sensitivity, with $b > 2$ in Eq. 1, (and with linear response to SM), like in the case of a positive feedback regulated systems9, where SM stimulates its own production. Such systems, typical for AHLs production, can rapidly drive SM production to very high concentrations and thereby increasing substantially the costs. This could be detrimental for a bacterium, especially if the cost per SM is high, like in the case of peptide ComX. To prevent this scenario Bacillus omits SM feedback loop thereby lowering the ultra-sensitivity (to $b = 2$ in Eq. 1) in SM production. The partial loss of ultra-sensitivity in SM production is, however, compensated by the ultra-sensitive response ($n = 2.3$, Eq. 2). Therefore, by distributing the ultra-sensitivity between the encoder and decoder, the ComQXPA communication system yields a response that has a steep enough transition from minimum to maximum response level to function as a true quorum sensing communication system. This ultra-sensitive and economic quorum sensing system, used by the entire cell population, relies on the oxygen concentration to adjust the signal production to the growth rate and the cell density.

**Methods**

**Bacterial strains.** Strains used in this study are listed in Supplementary Table 1. To obtain strain BM1042 the PS-216 srfA-cfp (cat)55 was transformed with plasmid pKB72 isolated from E. coli strain KTB360151. To obtain the strain BM1297, PS-216 srfA-cfp (cat)55 was transformed with the DNA isolated from strain DL594 (amyE::FomQX::yfp (spec))56. The strains are available from the authors upon request.

**Bacterial growth in batch fermentor system.** To measure concentration of the signal molecule (SM), i.e., ComX, and response to the signal molecule (RM), i.e., srfA promoter activity, during growth of B. subtilis the Minifors (Infors, AG, Switzerland) fermentor system operating in batch mode was deployed. Overnight cultures were grown in competence medium55 (CM) supplemented with L-histidine, L-leucine, and L-methionine (50 μg mL$^{-1}$). Prior to fermentor inoculation, the cells were washed twice by replacing the spent medium of overnight culture with SS buffer58. Cell suspension was inoculated into 1.2 L of fresh CM (2% inoculum). Incubation was performed at 37 °C with mixing at 700 rpm and the closed off air flow. In instances where we tested O$_2$ impact on SM concentration the medium was saturated with O$_2$ by ensuring a supply of compressed air at a flow rate of 1 L min$^{-1}$. The dissolved O$_2$ was measured using the O$_2$ polarized electrode InPro 6820 (Mettler Toledo). No pH correction was performed or anti foaming agents were added.

**Biosensor based quantification of signal molecule (ComX) concentration.** We have developed a biosensor-based method to measure the concentration of SM (ComX) in the spent medium of B. subtilis at different growth times. The biosensor BD2876 (Supplementary Table 1) does not produce SM (ComX), because it lacks the essential enzyme ComQ that is required for ComX maturation. However, it can respond to exogenous ComX via the receptor/response regulator pair ComP/ComA that trigger the srfA promoter during the incubation of the biosensor in the medium containing ComX. The activity of this promoter can be measured via β- galactosidase reporter and correlated to ComX concentration. Biosensor test provides two data sets: (i) β- galactosidase activity as a function of relative SM concentration in spent media (unknown concentration of ComX) and (ii) β-
galactosidase activity as a function of known SM concentration. After fitting these data to the Hill equation (Eq. 2) we obtained a sample and the calibration curve, respectively. The concentration of SM in spent medium (in duplicate) was calculated based on the comparison of the two curves (for details see Supplementary Methods, ComX concentration measurements) by considering the linear part of the sigmoid curve. In this way a possible saturation and consequent underestimation of ComX concentration was avoided.

ComX concentration was determined by measuring the growth of 

E. coli PS-216 (ΔcomP) strains. To determine the fitness cost of signal production we compared the growth dynamics of signal negative 

E. coli PS-216 (ΔcomP) strains with the growth curve of 

E. coli PS-216 strain containing a plasmid (pBC1926) with the IPTG inducible comQ gene19, under non-inducing and inducing conditions. (i) Ten microliters of overnight culture of the tagged strain were transferred to each of the coated wells (5 for tagged and 5 wells for non-tagged fluorescence microscopy (see, Gene expression evaluation by single cell fluorescence microscopy, Co-localization analysis).

Gene expression evaluation by single cell fluorescence microscopy. The gene expression evaluation of 

E. coli cells tagged with yfp and cfp was based on the previously set protocol25. Briefly, diagnostic slides (10 wells/6 mm) were coated by transferring 2.5 µl of a 0.05% (w/v) poly-L-lysine (Sigma) solution (0.9% NaCl) to remove the unattached cells and excess solution at room temperature until water evaporated. The samples of approximately 1 mL of the culture samples of fluorescently tagged and non-tagged 

E. coli PS-216 strains were collected at selected time intervals during the incubation in batch fermenter system. After washing the cells by SS buffer, ~15 µl of cell suspension was transferred to each of the coated wells (5 for tagged and 5 wells for untagged strains) of a diagnostic slide and incubated for 15 min at room temperature to allow cells to adhere to the wells. Afterwards, the slide was rinsed with physiological solution (0.9% NaCl) to remove the unattached cells and excess fluid was dispersed. To reduce photo-bleaching 2 µl of the SlowFade Gold antifade reagent (Thermo Fisher Scientific, Inc., USA) were added to each well and immediately taken for observation under Axiocam Epi fluorescence microscope (Zeiss, Göttingen, Germany). Differential interference contrast (DIC) and fluorescence images were observed (objective x100, NA 1.4, Zeiss) and recorded with a coupled MRm Axiocam camera (Zeiss) operating in 2 x 2 binning mode. The filter-sets used for fluorescent contrast were CFP (450/495 HE) and YFP (520/570 HE) and the exposure time of the time points at least 1000 bacteria were examined for their fluorescence.

For quantification and flat field correction the fluorescence sample microcopy images were firstly normalized on the sodium fluorescein standard. To take into account also potentially non-fluorescent cells, the DIC images of the same field of view were taken simultaneously with the fluorescent images. The normalized fluorescence intensity of single cells is then extracted by combining the location information about all cells in the DIC images with the fluorescence images via custom made ImageJ script (for the script refer to previously set protocol25). In order to take into account the autofluorescence of the cells, the same procedure was applied to the images of control samples with the non-fluorescently tagged cells. Then, by using OriginPro software (OriginLab, Massachusetts, USA) we were able to obtain the lognormal distribution of fluorescent marker (i.e., Yfp or Cfp). The procedure is however not trivial, as the fluorescence intensity of tagged cells contains besides the fluorescence intensity of the marker also autofluorescence that can be subtracted using the CFP channel. Although the normalization intensity contributions are independent and in this case the distribution of fluorescence intensity of the tagged cells is the convolution of distribution of the
fluorescence intensity of the marker (i.e., Yfp or Cfp) and the distribution of background fluorescence intensity (autofluorescence). To extract the parameters of unknown distribution of fluorescent marker, we fitted the distribution of fluorescence intensity of tagged cells by convolution of background fluorescence intensity (autofluorescence) and unknown lognormal fluorescent marker distribution. For details refer to our previous study. The outliers in Yfp or Cfp distributions (i.e., hyperproducers) were determined using Grubbs’s statistical test in OriginPro software, which is based on two-sided student t-test, by setting the significance level to 0.05.

**Co-localization analysis.** To spatially match fluorescence intensity of αnA–αp with comQ-yfp simultaneously expressed in the same cells the custom script was written and run in OriginPro. The same procedure was applied also to the control, i.e., the cells without fluorescence markers (autofluorescence). From the two datasets two correlation graphs were plotted (OriginPro) and linear Pearson correlation coefficient determined. As the correlation coefficient of the labelled cells also encompasses the correlation of autofluorescence, one cannot directly evaluate the correlation of Cfp with Yfp. Therefore, one has to compare the correlation coefficient of the control cells with fluorescently labeled cells. To further aid the interpretation of the correlation we simulated the intensities of labeled cells with an assumption that no correlation in Cfp and Yfp is present. The fluorescence intensities calculated on the basis of lognormal distributions obtained from the gene expression evaluation with single cell microscopy (see Gene expression evaluation with single cell fluorescence microscopy) were randomly added to the fluorescence intensities control cells for each fluorescence channel independently. The correlation coefficient of this set of data that represented the no correlation case of Cfp with Yfp was then used for comparison with the correlation coefficients of labeled and control cells.

**Data modeling and simulation of the response to the signal molecule and signal molecule production.** The analytical form of the model equation for response per cell to the signal during bacterial growth in the fermentor batch system (Eq. 3, eq S11) was derived and fitted to the experimental data by the help of Wolfram Mathematica 11.0 (Wolfram Research, Inc.). Simulation of various modes of normalized response as a function of bacterial density was performed in OriginPro software (OriginLab, Massachusetts, USA). The fitting of experimental data of signal molecule (SM) concentration as a function of the bacterial density and normalized response, W(SM), as a function of SM concentration was performed using ametric function (Eq. 1) and Hill equation (Eq. 2), respectively. The fitting by logistic equation of growth curves (eq S12) was performed in OriginPro as well. To minimize reduced $\chi^2$ that was used as a criterion for goodness of fit, the Levenberg–Marquardt method was applied (OriginPro). The lowest level of response (LLR) was determined from the graph of the normalized response as a function of SM (ComX) concentration as the first significantly positive value of the response (i.e., the lower 95% confidence level > 0) in the direction of increasing ComX concentration.

**Statistics and Reproducibility.** Statistical analysis was performed using the OriginPro (OriginLab, Massachusetts, USA) or Wolfram Mathematica 11.0 (Wolfram Research, Inc.). Unpaired Student’s T-test, two-sided, was used to calculate the statistical significance of data sets. A P value of less than 0.05 was considered statistically significant. The model fits are shown together with 95% confidence level. The measure of the quality of fits was reduced $\chi^2$ and $R^2$. The error of obtained values of fitting parameters is the standard error (SE), unless stated otherwise. To determine the number of hyper-expressing cells, Grubbs’s test was used. If experiments were performed in microtiter plates, at least 8 wells were used as replicates within a biological replicate. In experiments with larger volumes at least two Erlenmeyer flasks were used within an experiment. If experiments performed all our experiments in at least three biologically independent experiments. In microplate assays, we performed statistical analysis by using modified $Z$-score to exclude the outliers. The lower limit of detection (LOD) for biosensor assay for ComX concentration measurements was calculated from calibration curves and was defined as the minimal ComX concentration that induces the response in the biosensor BD2876 that significantly differs from the response of the blank. Therefore, the LOD was determined as ComX concentration at the response that equals to the response of the blank + 3 standard deviations of the response of the blank.

**Data availability** The authors declare that the relevant data supporting the findings of this study are available in the article and its Supplementary Information files, or from the corresponding author upon request.

**Code availability** The ImageJ macro code for extracting the fluorescence intensities of B. subtilis PS-216 (ΔcomQ P$_{comX}$Yfp) and the OriginC code for matching the fluorescence intensities of Cfp and Yfp in B. subtilis WT PS-216 (comQ-yfp, αnA-αp) imaged by fluorescence microscopy are freely available on Github by https://doi.org/10.5281/zenodo.4205585 and https://doi.org/10.5281/zenodo.4206230, respectively.

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