Frequency modulation of a bacterial quorum sensing response

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In quorum sensing, bacteria secrete or release small molecules into the environment that, once they reach a certain threshold, trigger a behavioural change in the population. As the concentration of these so-called autoinducers is supposed to reflect population density, they were originally assumed to be continuously produced by all cells in a population. However, here we show that in the α-proteobacterium Sinorhizobium meliloti expression of the autoinducer synthase gene is realized in asynchronous stochastic pulses that result from scarcity and, presumably, low binding affinity of the key activator. Physiological cues modulate pulse frequency, and pulse frequency in turn modulates the velocity with which autoinducer levels in the environment reach the threshold to trigger the quorum sensing response. We therefore propose that frequency-modulated pulsing in S. meliloti represents the molecular mechanism for a collective decision-making process in which each cell’s physiological state and need for behavioural adaptation is encoded in the pulse frequency with which it expresses the autoinducer synthase gene; the pulse frequencies of all members of the population are then integrated in the common pool of autoinducers, and only once this vote crosses the threshold, the response behaviour is initiated.
ar-reaching behavioural changes in bacterial populations are often initiated as a reaction to small molecules that the cells themselves produce and release into their environment. These molecules accumulate while the population grows and, once they reach a certain threshold, trigger changes in gene expression leading to, e.g., bioluminescence, virulence or biofilm formation. As the respective molecules are self-produced, they are termed autoinducers, and the phenomenon was initially referred to as autoinduction; as the triggered behaviours were assessed to be effective only when performed by a large enough group and the autoinducer concentration to indicate when this sufficient population size—the quorum—is reached, the far more popular term for the process now is 'quorum sensing'.

Based on their ascribed role as indicators of population density, autoinducers were originally assumed to be continuously produced by all cells in a population. However, over the past decade several cases of cell-to-cell heterogeneity in autoinducer synthase or precursor gene expression have been reported: e.g., in expression of the Listeria monocytogenes agr operon encoding the autoinducer precursor AgrD, and in expression of the autoinducer synthase genes ahlI in Pseudomonas syringae, tral and ngrI in Sinorhizobium fredii, and sinI in Sinorhizobium meliloti. Furthermore, there is indication of heterogeneity in AHL synthase gene expression in Pseudomonas putida. Both the precise nature of these heterogeneities—whether they represented stable subpopulations with distinct expression levels, or rather variations over time—and their molecular origins remained unclear, but their observation nevertheless indicated that the model of constitutive autoinducer production is not universally valid.

Moreover, both biotic factors like nutrient availability or stress and abiotic factors like diffusion or flow have long been known to affect autoinducer-mediated regulation. For instance, luciferase production and bioluminescence in Aliviibrio fischeri is delayed via catabolite repression of the autoinducer receptor gene in presence of glucose. Similarly, autoinducer production and target gene expression in Erwinia carotovora are altered by the type of carbon source provided, and activation of the Pseudomonas aeruginosa las and rhl quorum-sensing systems likewise varies depending on growth conditions. It has therefore been repeatedly acknowledged that the term 'quorum sensing' represents an oversimplification and should be used with full appreciation of the many environmental factors influencing it. Even functions alternative or complementary to cell-density sensing were proposed, ranging from simple sensing of diffusion rates to the integration of different cues like cell density, clustering and diffusion, or nutritional status and stress. Here we show how in the α-proteobacterium S. meliloti phenotypic heterogeneity in autoinducer synthase gene expression and physiological influences on quorum sensing are linked in a collective decision-making process in which the first represents the key for integration of the latter.

**Results**

Stochastic pulsing in a canonical LuxR-LuxI-type quorum sensing system. S. meliloti is a widely-studied model organism for symbiosis with leguminous plants, but like other rhizobia it can also be found free-living in the soil. It has a canonical Gram-negative quorum sensing system homologous to the A. fischeri LuxR-LuxI system where LuxI is the synthase producing N-acyl homoserine lactones (AHLs) as autoinducers and LuxR is the cognate receptor, triggering the response upon AHL binding. The Sin system in the Sin system produces long-chain AHLs that are sensed by the LuxR-type regulator ExpR. However, the Sin system has an additional player: SinR, a LuxR-type regulator that, according to our analysis, has a degenerated AHL binding motif. Transcription of sinI strictly depends on SinR and is enhanced by binding of ExpR-AHL to the sinI promoter, giving rise to a positive feedback loop; at very high AHL concentrations, ExpR-AHL represses sinI transcription.

As indicated above, expression of sinI in wild-type S. meliloti has been found to show strong cell-to-cell variation in fluorescence levels from a sinI promoter-fluorophore gene fusion. To examine whether this variation reflects heterogeneity already present upstream in the regulatory network or rather

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**Fig. 1** sinI expression is realized in stochastic pulses. a Simplified sketch of the regulatory network controlling AHL synthase gene expression in S. meliloti. b Fluorescence intensities from two sinI promoter-reporter gene fusions within individual cells determined by microscopy. Pooled data from 10 (wt) and 12 (expR−) colonies, respectively, imaged on 3 different days. Total number of cells analysed: N = 1190 (wt), 1287 (expR−). rS, Spearman’s correlation coefficient; P (two-tailed) <0.0001 for both data sets. See Supplementary Fig. 2a for raw images and details on the construct. Supplementary Fig. 2b for confirmation from an alternative construct. c (Top) Phase contrast and fluorescence images from a microscopy time lapse of an expR− microcolony carrying a Psint-mVenus fusion, and the ‘red fire’ lookup table applied to the fluorescence images. Data representative of 9 colonies imaged on 3 different days; scale bar, 2 μm. (Bottom) For cells #5 and #6, both total and mean fluorescence intensities first increase. Total fluorescence then drops with cell divisions and stays almost constant in between, while mean fluorescence constantly decreases with cell growth. Gene expression rate is calculated as the change in mean fluorescence intensity over time; peaks in gene expression rate are broadened by the regression involved in the calculation. See ‘Methods’ and Supplementary Fig. 3a for details and further examples.
stochastic processes inherent to *sinI* expression, we first generated strains carrying two identical copies of the *sinI* promoter fused to two different fluorophore genes. In these strains, upstream heterogeneity should affect both reporters to a similar degree within individual cells, whereas stochastic events during *sinI* expression should affect the two fusions independently and thus lead to uncorrelated variations. Analysis of wild-type and *expR*– strains carrying such constructs by microscopy snapshots (Fig. 1b and Supplementary Fig. 2a) and flow cytometry (Supplementary Fig. 2b) showed a considerable fraction of non-fluorescing cells in both backgrounds. Furthermore, in some wild-type cells variation in fluorescence affected both reporters to a similar extent, whereas other wild-type cells displayed highly diverging intensities from the two reporters. In *expR*– cells, activation of the two promoter-fluorophore gene fusions was almost entirely uncorrelated, with most of the fluorescing cells showing fluorescence from either one or the other reporter. The overall low degree of correlation indicates that heterogeneity mainly stems from stochasticity inherent to *sinI* expression.

To further explore this stochasticity, we next followed *expR*– microcolonies carrying a single *sinI* promoter-*mVen*s fusion via time-lapse microscopy (Fig. 1c, Supplementary Movie 1). Here, cells were usually dark, and when fluorescence appeared, it did so not in a coordinated fashion comprising the whole colony, but only in individual cells, and only temporarily. However, fluorescent reporters are stable proteins, and their levels thus reflect both current and past expression; to more accurately examine changes in *sinI* expression over time, we therefore calculated its expression rate adapted from Locke et al.

### A regulatory system based on very low odds

As intrinsic stochasticity was manifest both in the wild type and the *expR*– strain, and was even more pronounced in the latter, we next investigated its most likely source—the essential transcription activator SinR—in the *expR*– background. Expression of *sinR*, when assayed with a *sinR* promoter-*mCherry* fusion via microscopy, appeared rather weak and homogeneous (Fig. 2a), consistent with the above-drawn conclusion that heterogeneity in *sinI* expression does not originate upstream in the regulatory network. However, in vivo protein stability assays yielded a half-life of only about 3 min for a Flag-tagged SinR fusion protein when produced from the chromosomal *sinR* promoter (Fig. 2b, Supplementary Fig. 4a, b); and single-molecule microscopy of fixed cells carrying an *mScarlet-I-sinR* translational fusion at the chromosomal locus indicated that—after background subtraction—only about 10% of cells in a population, at a given time, have *mScarlet-I-SinR* spots (Fig. 2c; Supplementary Fig. 4c). Furthermore, when examining the effects of Flag-tagged SinR and *mScarlet-I-SinR* on the *Psir-mVen*s reporter construct, the fusion proteins produced much higher fractions of fluorescing cells in flow cytometry measurements than native SinR (Supplementary Fig. 4d), suggesting that the latter is even less stable and/or abundant than its tagged versions. Such low protein abundance might seem unusual; however, a half-life of only 2 min has been reported for the *Agrobacterium tumefaciens* LuxR-type regulator TraR in absence of autoinducer,

### Fig. 2 SinR scarcity is a key factor in *sinI* expression pulsing

**a** (Left) Raw phase contrast and fluorescence microscopy images of a strain carrying a *sinR* promoter-*mCherry* fusion, and the ‘green fire’ lookup table applied to the fluorescence image. Scale bar, 2 µm. (Right) Frequency distributions of mean mCherry intensities per cell and corresponding Gaussian fits suggest homogeneous *sinR* expression in the *expR*– strain. Pooled data from snapshots of 7 and 6 colonies, respectively, imaged on 2 different days. 

**b** Relative abundance of Flag-tagged SinR (F-SinR) after chloramphenicol treatment in 1-min intervals determined by Western blot analysis and a one-phase exponential decay fit to the data. Data, means ± standard deviations of 3 biological replicates. 

**c** (Left) Cut-out from a single-molecule microscopy snapshot of an *expR*– strain expressing an *mScarlet-I-sinR* fusion from the chromosomal *sinR* promoter. Arrows mark fluorescing spots. (Right) Bar plots indicating the fraction of cells with fluorescing spots in this strain and the corresponding control strain lacking the fluorophore gene in 3 biological replicates; bars represent means ± standard deviations, open circles represent individual data. Statistical test, two-tailed unpaired *t*-test with Welch’s correction. ns, not significant; *P* = 0.1149. Total number of cells analysed: *N* = 2293 (*mScarlet-I-sinR*), 1670 (control).

**d** Manipulation of (*mScarlet-I*) *sinR* transcription levels yields corresponding patterns of (left) the fraction of cells displaying *mScarlet-I-SinR* spots in single-molecule microscopy and (right) *Psir-mVen*s expression pulse frequencies in time-lapse fluorescence microscopy. *Psir*, promoter mutation resulting in reduced transcription; native, native promoter; *nurR*+, native promoter while overproducing its transcription activator NurR. Bar plots indicate means ± standard deviations and individual data from (turquoise) single-molecule microscopy (SMM) performed in 3 biological replicates and (blue) time-lapse fluorescence microscopy of 9 colonies imaged on 3 different days. Statistical tests, Welch’s ANOVA tests with post hoc Dunnett’s T3 multiple comparisons test. ns, not significant; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001. Multiplicity-adjusted *P* values: *Psir* vs. native 0.1119, *Psir* vs. *nurR*+ 0.0012, native vs *nurR*+ 0.0217 for SMM data, 0.0290, <0.0001, <0.0001 for pulse data, respectively. Total number of cells analysed: *N* = 1260 (*Psir*), 1142 (native), 1158 (*nurR*+), for SMM data, 3411, 2900, 2440 for pulse data, respectively.

To test whether SinR scarcity is a determinant in *sinI* expression pulsing, we then generated two strains with slightly reduced and slightly increased *sinR* expression levels, respectively; the former by introducing a mutation into the *sinR* promoter interfering with binding of its transcription activator NurR, the latter by overexpressing *nurR* from a plasmid. Single-molecule
microscopy confirmed that the fraction of cells displaying mScarlet-I-SinR spots in the two strains was altered by the manipulations as intended, and time-lapse microscopy indeed yielded about 7-fold reduced and 3-fold increased sinI expression pulse frequencies, respectively (Fig. 2d and Supplementary Fig. 5a). When we repeated the analysis with different thresholds for what is considered a pulse, absolute pulse frequencies of course changed, but the relative differences between the strains remained (Supplementary Fig. 6). Furthermore, the fraction of fluorescent cells in flow cytometry measurements—a proxy for pulse frequency, as, e.g., a higher frequency over time in individual cells should produce a higher fraction of fluorescent cells in a population at a given time—was altered in a corresponding fashion (Supplementary Fig. 5b); nurR overexpression in a sinR+ or sinR− promoter mutation background in turn did not affect this fraction (Supplementary Fig. 5c). In contrast, direct overproduction of (mScarlet-I)-SinR from a plasmid not only abolished heterogeneity in fluorescence both from the mScarlet-I-SinR and the PsinI-mVenus fusion, but also greatly increased fluorescence intensities (Supplementary Fig. 5d), disrupting the otherwise stochastic regulatory system. Thus, scarcity of SinR is indeed a determining factor for sinI expression in a pulsatile rather than a continuous fashion.

**Pulse frequency fine-tuned by physiological factors.** As we were able to modify PsinI-mVenus pulse frequencies artificially, we next explored whether pulse modulation also occurs physiologically. As mentioned above, effects of various biotic and abiotic cues on quorum sensing are well-established 3,12–14,26, and pulse modulation might well represent a mechanism for integrating physiological information on the dynamic scale. Population-level studies in *S. meliloti* had shown sinI expression to be enhanced by phosphate starvation 29, to be decreased by elevated levels of the cyclic-di-GMP 36, and, as mentioned above, to be enhanced by ExpR-AHL-mediated positive feedback in the wild type 29. When we examined the effects of the respective growth conditions and genetic backgrounds at the single-cell level, phosphate starvation indeed increased sinI expression pulse frequency, the fraction of PsinI-mVenus fluorescenting cells, and the fraction of cells with mScarlet-I-SinR spots in the expR− background compared to rich growth conditions (Fig. 3a, Supplementary Fig. 7a, b); and an expR− strain incapable of producing detectable amounts of c-di-GMP (dgc0) 36 likewise showed increased sinI pulse frequency, fraction of fluorescenting cells, and cells with mScarlet-I-SinR spots, while an expR− strain producing elevated levels of c-di-GMP (pde0) (Supplementary Fig. 8) showed the reverse phenotype.
namely reduced pulse frequency, a smaller fraction of fluorescing cells, and fewer cells with mScarlet-I-SinR spots (Fig. 3b, Supplementary Fig. 7c, d).

In the wild type capable of ExpR-AHL-mediated positive feedback, pulse frequency and flow cytometry frequency were even raised ~10-fold compared to the expR− strain, to about 0.28 pulses per hour and cell (Fig. 3c, Supplementary Fig. 7e, f, Supplementary Movie 2). However, presence of expR did not increase the fraction of cells with mScarlet-I-SinR spots correspondingly (Supplementary Fig. 7g). Instead, we could detect a His-GB1-SinR-dependent supershift of the sinI promoter in electrophoretic mobility shift assays (EMSA) in the presence of His-ExpR-AHL, but no shift by purified His-GB1-SinR alone even at high concentrations (Fig. 3c). First, this observation suggests a very low binding affinity for SinR alone—too low to be detectable by our assay, and a feature that very likely adds to the stochasticity of the system (see Supplementary Fig. 7h for in vivo detectable by our assay, and a feature that very likely adds to the

Gene expression due to growth conditions or c-di-GMP levels (Fig. 3d) – about 2-fold; flow cytometry data shows a similar trend, with a 65-fold change in the fraction of fluorescing cells, and a 6-fold change in intensity (Fig. 3f; Supplementary Table 1). Hence, regulation of sinI expression primarily happens through frequency modulation, and amplitude modulation plays a minor role at best.

Response dynamics determined by pulse frequency. The differences in sinI expression pulse frequency in single cells should in turn impact behaviour on the level of the group, as they will affect the overall AHL production rate of the population, and, consequently, the velocity with which autoinducer concentrations in the environment reach the threshold to trigger the quorum-sensing response. To test this rationale, we followed the wild-type colonies that had shown frequency-modulated pulsing in sinI expression due to growth conditions or c-di-GMP levels (Fig. 3d) for 10 or more hours after they became three-dimensional. At this stage we assessed fluorescence no longer at the single-cell level, but as mean fluorescence intensities of the whole colony from a wgaA promoter-mCerulean fusion—the wgaA promoter regulates expression of a gene cluster involved in production of galactoglucon, an exopolysaccharide (EPS) that plays an important role in S. meliloti colony expansion and sliding motility39–41 and that is a central part of the organism’s quorum sensing response38–40. As expected, response onset in strains displaying higher sinI expression pulse frequencies could be observed several hours earlier and at smaller colony sizes (Fig. 4a, Supplementary Fig. 12a–f) than in colonies with lower pulse frequencies. However, as phosphate starvation and c-di-GMP levels might impact EPS production not only via quorum sensing (e.g., Supplementary Fig. 12c), we furthermore sought to isolate AHL production-response-dynamics from other regulatory networks in the organism. To this end, we made use of the constructs with artificially altered sinR expression levels and sinI expression pulse frequencies (Fig. 2d, Supplementary Fig. 5), harvested their supernatants at various optical densities and added them to the growth medium of an S. meliloti AHL indicator strain, assuming that differences in the quorum-sensing response of the latter should then solely result from differences in the amount of AHLs in the respective supernatants (Supplementary Fig. 12g, h). Even in this decoupled system, response curves staggered according to pulse frequencies of the donor strains (Fig. 4b). Thus, when adapting to changes in environment or lifestyle, S. meliloti cells adjust AHL synthase gene expression pulse frequency, resulting in response onset at larger or smaller cell numbers (Fig. 4c).

Discussion

The stochastic pulsing in sinI expression reported here resembles the pulsatile activity of several stress-responsive transcription factors in Saccharomyces cerevisiae43–45, E. coli46 and other alternative sigma factors in Bacillus subtilis47,48, and the short gene expression bursts from the uninduced lac promoter in E. coli47,48. Similar activity profiles have been furthermore described for higher eukaryotes including mammals, and the terms ’transcription pulse’ and ’transcriptional burst’ are sometimes used synonymously to describe such phenomena49,50. In contrast, Levine et al. define pulsing as a phenomenon ”generated by genetic circuits that activate and deactivate key regulators and modulate pulse characteristics, such as frequencies and amplitudes”, whereas ”transcriptional bursting […] results from the stochastic nature of gene expression”51. However, judging from

A linear correlation between key activator abundance and pulse frequency. Based on our hitherto cumulated data, we furthermore sought to analyse the relationship between mScarlet-I-SinR abundance and sinI expression more deeply. Both the high degree of stochasticity observed in sinI expression (Fig. 1b) and the homogeneity observed in sinR expression (Fig. 2a) had already indicated that heterogeneity in sinI promoter activity does not originate upstream in the regulatory network, i.e., from cell-to-cell differences in sinR expression. Closer examination of the single-molecule microscopy data from all mScarlet-I-sinR strains (with exception of the overexpression strain) supports this conclusion, as it suggests that mScarlet-I-SinR spots do not contain higher-order multimers, but only one or two functional mScarlet-I molecules, and thus very likely only one or two SinR molecules (Supplementary Fig. 10). Moreover, all data gathered in the expR− background indicates a linear correlation between the fraction of cells displaying mScarlet-I-SinR spots and frequency of sinI expression pulses (Fig. 3e; Supplementary Table 1). In contrast, pulse amplitude and fluorescence intensity as its corresponding property in flow cytometry data do not appear to correlate with SinR abundance. When plotting pulse amplitude against frequency for all expR− and wild-type data, frequency increases about 91-fold over the whole data set, while amplitude varies only about 2-fold; flow cytometry data shows a similar trend, with a 65-fold change in the fraction of fluorescing cells, and a 6-fold change in intensity (Fig. 3f; Supplementary Table 1). Hence, regulation of sinI expression primarily happens through frequency modulation, and amplitude modulation plays a minor role at best.
our findings on sinI expression, the genetic circuit and the stochastic nature of gene expression are not always clearly distinguishable; rather, stochasticity is an integral part of the *S. meliloti* Sin system.

For instance, when comparing *sinI* expression pulsing and the pulsatile activity of the *B. subtilis* stress response sigma factor σB\(^{32}\), both are asynchronous and share features like variability in amplitude and frequency modulation by physiological factors. On the other hand, they differ fundamentally with respect to the stochasticity involved, indicative of the disparate mechanisms underlying the two phenomena: Whereas *S. meliloti* cells carrying two different *sinI* promoter-fluorophore gene fusions displayed highly diverging intensities from the two reporters both from cell to cell and within individual cells (Fig. 1b, Supplementary Fig. 2), fluorescence intensities of analogous *sigB* promoter-fluorophore fusions in *B. subtilis* only varied from cell to cell, but were highly correlated within cells\(^{32}\). Moreover, activity of the *sigB* promoter was also highly correlated with activities of other σB\(^{32}\)-regulated promoters in the respective cells\(^{32}\). Stochasticity in σB activity is thus restricted to whether or not, in a given cell at a given time, a pulse is initiated. This decision according to Locke et al. is triggered by a phosphoswitch, i.e., fluctuations in the ratio of phosphatases and kinases acting on the σB anti-anti-sigma factor that set off time-delayed positive and negative feedback loops, with the positive feedback first turning stochastic *sigB* promoter activation into a cell-wide pulse, and the negative feedback subsequently terminating it\(^{32}\).

In contrast, *sinI* expression pulses begin and end without feedback loops. Instead, they very simply stem from instability and scarcity of the key activator SinR (Figs. 2 and 3e), and very likely also from low binding affinity of SinR to the *sinI* promoter (Fig. 3c). Together, these biochemical properties of SinR yield a very low probability for a *sinI* transcription event, and a short duration of such an event if it does occur. With respect to the underlying mechanism, pulsing in *sinI* expression thus is a reversed image of stochastic gene expression from uninduced *lac* promoters in *E. coli*: For a reduced version of the promoter comprising only the O\(_1\) and O\(_2\) operators, Yu et al. reported short transcriptional bursts with a mean frequency of 1.2 events per cell cycle\(^{47}\), and Cai et al. reported similarly brief bursts with a mean frequency of 0.11 events per cell cycle for the wild-type *lac* promoter comprising all three operators O\(_1\), O\(_2\) and O\(_3\)\(^{38}\). In both cases, the transcriptional bursts were attributed to stochastic and brief dissociation of the *lac* repressor LacI from the respective promoters. Furthermore, both cell-to-cell and within-cell heterogeneity was observed in a seminal study by Elowitz et al. from two promoter-fluorophore gene fusions in which the identical synthetic promoters contained the O\(_1\) operator\(^{39}\). However, stochasticity in this case was much less prominent than observed for the two analogous *sinI* promoter-fluorophore gene fusions (Fig. 1b, Supplementary Fig. 2), probably because the synthetic promoter\(^{32}\) does not enable the DNA loop formation crucial for enhanced repression by LacI\(^{33}\), thus making dissociation events more likely. Based on differences in repression of the *lac*
promoter versions\textsuperscript{53}, and on differences in burst frequencies\textsuperscript{47,48}, one would therefore expect stochasticity to be more prominent for the \( \text{O}_2 \) & \( \text{O}_2 \) lac promoter version used by Yu et al., and even more so for the wild-type version comprising all three operators studied by Cai et al.

Due to the very low probability for SinR binding to the \textit{sinI} promoter, it is impossible to predict whether or not a given cell at a given time will experience a \textit{sinI} expression pulse. Nevertheless, \textit{sinI} expression is by no means random or arbitrary in the sense of ‘happening without cause or reason’—over a large enough population, the fraction of cells with a SinR-\textit{sinI} promoter complex and ensuing \textit{sinI} expression is clearly defined by abundance of SinR, and by abundance of ExpR and AHLs affecting SinR binding affinity (Fig. 3). Similarly, the term ‘noise’, albeit widely used as a synonym for stochasticity\textsuperscript{31,34,55}, does not seem appropriate in this context, since it has connotations of mere statistical fluctuations. In contrast, the Sin system is based on low probabilities, and without them, regulation of \textit{S. meliloti} quorum sensing would be entirely different: A higher binding affinity of SinR to its promoter, for instance, with everything else unchanged, would considerably increase \textit{sinI} expression rate, and the same is of course true for higher SinR abundance (Supplementary Fig. 5d); both would thus strongly increase AHL production in the population and accelerate quorum-sensing dynamics (Fig. 4b). If, on the contrary, the dynamics were to be preserved, a steady \textit{sinI} transcription would have to be compensated for by, e.g., a reduced \textit{sinI} translation rate, a reduced AHL production rate, and/or a reduced sensitivity of the AHL receptor to autoinducers. The Sin system thus represents a probabilistic switch operating at low odds, and the setup of this switch furthermore allows for the integration of physiological factors, as these either fine-tune abundance of SinR (Fig. 3a, b), or its binding affinity (Fig. 3c), and thereby modulate \textit{sinI} expression pulse frequency.

The connection between environmental cues and quorum sensing dynamics per se is not novel: Population-level studies in \textit{S. meliloti} had already shown \textit{sinI} expression to be affected by the respective cues\textsuperscript{29,36}, just as—for instance—population-level studies in \textit{A. fischeri} had shown luciferase production and bioluminescence to be delayed via catabolite repression\textsuperscript{16–20}. Indeed, Fuqua et al. emphasized the role of physiological factors when first proposing the term ‘quorum sensing’, stating that, in addition to the sufficiently high cell density for autoinducers to accumulate to a threshold concentration, “first, some external environmental signal other than an autoinducer must be perceived”\textsuperscript{2}. Dunn and Stabb reasoned that “by embedding quorum signalling with […] regulatory systems [like catabolite repression], bacteria are able to modulate the production of autoinducers such that their concentration reflects not only cell density but also specific parameters of their environment”, and that target genes are thus regulated “not always with direct correlation to population numbers”\textsuperscript{3}. And after examining the activity of \textit{P. aeruginosa las} and \textit{rhl} quorum sensing systems under 46 growth conditions, Duan and Surette even concluded that “no correlation could be established between cell densities and the activation of quorum sensing expression […], indicating the absence of a specific cell density as a prerequisite for quorum sensing activation”\textsuperscript{22}.

Similarly, we found that the onset of the quorum-sensing response in \textit{S. meliloti} populations is triggered at smaller or larger cell numbers depending on the physiological state of the individual cells (Fig. 4a, Supplementary Fig. 12), implying that the process of autoinducer production and sensing in \textit{S. meliloti} is likewise not a simple matter of counting cell numbers as suggested by the analogy of the quorum. Since we furthermore found that the physiological state of the individual cells is encoded in their \textit{sinI} expression pulse frequency (Fig. 3), the process seems more comparable to a voting in a local community, or to the collective decision-making described for social insects, e.g., during selection of a new nest site by a swarm of honey bees\textsuperscript{56–60}. Whereas the vigour of a scout bee’s waggle dance is proportional to the quality of the potential nest site it has explored, convincing more bees to likewise visit that site and cast their votes, the pulse frequency with which an individual \textit{S. meliloti} cell expresses the AHL synthase gene carries information about its physiological state and need for behavioural adaptation. Even a similar amplification process appears to be involved, as the AHLs produced by one bacterium facilitate \textit{sinI} expression in its neighbours by increasing binding affinity of SinR to the \textit{sinI} promoter, should the neighbours experience a similar need for action and, thus, a similar increase in SinR abundance. Due to the common pool of autoinducers—comparable to a ballot box—the pulse frequencies of all members of the population are then integrated into the total AHL concentration; only if this vote crosses the threshold, the response behaviour is initiated.

As mentioned above, the phenotype heterogeneity has been reported not only for AHL synthase gene expression in \textit{S. meliloti}\textsuperscript{8}, but also for expression of the homologous genes \textit{ngr}I and \textit{tral} in its close relative \textit{S. fredii}\textsuperscript{61}, the homologue \textit{ahl} of \textit{P. syringae}\textsuperscript{62}, and for expression of the \textit{agr} operon encoding the quorum sensing system of \textit{L. monocytogenes}\textsuperscript{63}; in these studies, the respective quorum sensing-ON and -OFF fractions determined by microscopy snapshots or flow cytometry were also affected by environmental factors\textsuperscript{5–7}. It would be curious to see whether these heterogeneities represent stable subpopulations, or likewise result from asynchronous stochastic pulsing, thus making frequency modulation as described here a recurring mode for collective decision-making in bacterial quorum sensing.

\section*{Methods}

\subsection*{Media and growth conditions.} Rich media were used for strain construction and maintenance: lysogenic broth (LB) medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) for \textit{Escherichia coli} strains, tryptone-yeast extract (TY) medium (5 g/l tryptone, 3 g/l yeast extract, 0.4 g/l CaCl\(_2\) x 2 H\(_2\)O) for \textit{Sinorhizobium meliloti} strains.

If required for selection during \textit{E. coli} strain construction or for plasmid maintenance in \textit{E. coli} strains, kanamycin was added at 50 mg/l, gentamicin at 8 mg/l and ampicillin at 150 mg/l to solid media. For selection during \textit{S. meliloti} strain construction and for plasmid maintenance in \textit{S. meliloti}, streptomycin was added at 60 mg/ml, kanamycin at 60–200 mg/ml, and gentamicin at 40 mg/l. For liquid cultures, antibiotic concentrations were generally reduced by half if not indicated otherwise. Selection for sucrose sensitivity of \textit{S. meliloti} clones after double homologous recombination was carried out on LB agar containing 10% (w/v) sucrose\textsuperscript{64}.

Starter cultures for flow cytometry, microplate reader measurements, single-molecule microscopy and western blot analysis of Flag-tagged SinR were grown in 3 ml modified morpholinopropane sulfonate (MOPS)-buffered medium slightly adapted from ref.\textsuperscript{65} to exponential phase; the exact composition was 1x MOPS solution (10 g/l MOPS, 10 g/l mannitol, 3.93 g/l sodium glutamate, 0.246 g/l MgSO\(_4\) x 7 H\(_2\)O, pH 7.2, autoclaved), with CaCl\(_2\) (37 mg/ml, autoclaved), FeCl\(_3\) x 6 H\(_2\)O (10 mg/ml, filter-sterilized and stored at 4°C), oligo-elements (3 mg/ml H\(_2\)BO\(_3\), 2.23 mg/ml MnSO\(_4\) x 4 H\(_2\)O, 0.288 mg/ml ZnSO\(_4\) x 7 H\(_2\)O, 0.125 mg/ml CuSO\(_4\) x 5 H\(_2\)O, 0.065 mg/ml CoCl\(_2\) x 6 H\(_2\)O, 0.12 mg/ml NaMoO\(_4\) x 2 H\(_2\)O, filter-sterilized) and biotin (1 mg/ml, filter-sterilized and stored at 4°C) all added in a 1:1000 dilution, and K\(_2\)HPO\(_4\) (174 mg/ml, autoclaved and stored at 4°C) added in a 1:500 dilution. For experiments involving titration of \textit{sinI} expression levels, all starter cultures were grown in presence of gentamicin for plasmid maintenance. For main cultures, if not otherwise indicated, 3 ml fresh modified MOPS-buffered medium without antibiotics was inoculated from starter cultures to yield an OD\(_{595}\) of about 0.1–0.3 at harvest the next morning; when involving titration of \textit{sinI} expression levels, isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) was added at 0.5 mM. For phosphate starvation, overnight cultures were harvested, washed three times in MOPS-buffered medium without phosphate, resuspended in MOPS-buffered medium without phosphate, and incubated for 5 more hours.
E. coli strains were grown at 37°C. S. meliloti strains at 30°C. Conjugations were incubated at 30°C. Liquid cultures were grown in glass test tubes shaking at 200 rpm.

Strain construction. Cloning was performed in E. coli DH5α, and final constructs were verified by DNA sequencing. Plasmid transfer into S. meliloti was carried out by E. coli S17-1-mediated conjugation, and if integration into the S. meliloti genome via single or double homologous recombination was involved, the resulting strains were again verified by sequencing. Strains, plasmids and primers used are listed in Supplementary Tables 2–4. Details on strain constructions are given in the Supplementary Methods 1.

(Time-lapse) fluorescence microscopy. Starter cultures were prepared as described above. One millilitre of starter culture was harvested by centrifugation (4000 x g, 5 min, RT), and cells were either immediately resuspended in modified MOPS-buffered medium to an OD_{600} of 0.25, or first washed three times in 1 ml MOPS-buffered medium without phosphate (for phosphate starvation conditions, to remove residual phosphate) or in 1 ml MOPS-buffered medium containing 2 mM phosphate (for the corresponding rich growth conditions; and for extracellular strains, to remove accumulated AHLs). Cell density was adjusted via serial dilutions to an OD_{600} of 0.000025. Two to three hours before harvest, agarose pads made from modified MOPS-buffered medium containing 1.5% (w/v) molecular biology grade agarose (Eurobio) were poured in a 17×28 mm or 9 mm Frame Slide in situ polymerase chain reaction and hybridization slide chambers (Biorad); the smaller frame size was chosen for side-by-side comparison of phosphate starvation vs. rich growth conditions as these required different pad composition, and for comparison of wt, dgc0 and ΔexpR strains to avoid alteration of strain-specific quorum sensing response dynamics by diffusing AHLs. Prior to adding cells, pads were allowed to dry for ~8–12 min depending on temperature and air flow; then, 0.3 μl per cell suspension (OD_{600} of 0.000025) were spotted on the pads, yielding ~3–4 dozen single cells per spot. For the phosphate starvation condition and the corresponding control condition, three additional 0.3 μl spots of scavenger/indicator cells (OD_{600} of 0.01) were added at the corners of the NIS Elements image (to allow an ND Acquisition module, as described in van Vliet et al. 65: First, we performed a shading correction to correct for light intensity inhomogeneities in the NIS Elements image, as colonies then grew larger than the field of vision of the camera. In this case, stitching of the image stacks was performed immediately on phase-contrast images (15% overlap) using the NIS Elements software. Over the whole time-lapse experiment, focus was maintained using the Perfect Focus System (PFS). Furthermore, to facilitate focus maintenance, microscope and incubation chamber were preheated for at least 4–5 h, preferably even overnight.

Fluorophores were excited with lasers: mCherry from mCherrulan with a 445 nm CUBE Laser (Coherent Inc., USA) [excitation band pass (ex) 440/45 nm, beam splitter (bs) 650 nm, emission band pass (em) 520/76 nm, em 580 nm] and mCherry with a 561 nm Sapphire Laser (Coherent Inc., USA) (ex bp 562/40 nm, bs 593 nm, em bp 624/40 nm). Laser intensities, exposure times and EM gains were applied as follows: 3%, 600 ms, 100 for Pslm-mVenus; 5%, 600 ms, 100 for Ptp-mCherry; 8%, 1, 100 for Pslm-mCherry; 4%, 1, 100 for Pslm-mVenus; 25%, 1, 5 s and 100 for Pslm-mCherry. For the 2×2 images, settings were modified as follows: 0.5%, 2×2 binning, 1, 100 for Pslm-mVenus; 0.5% 2×2 binning, 600 ms, 150 for Psoy-mCherrulan. Conversion gain was always set to 1. Generally, excitation intensities and exposure times were chosen as low as possible to minimize phototoxicity.

Processing, segmentation, tracking and single-cell analysis of early (2D) time-lapse data. The NIS Elements software was used to crop image stacks to the maximum spatial extent of the colony and to the time period during which cells were growing in a single layer. Further processing was done with a combination of Schnitzcells version 1.1 63, Itlastik version 1.3.3.post134, and a custom-built Matlab (MathWorks, Natick, Massachusetts) programme. 65 The workflow closely follows the pipeline developed by van Vliet et al. 7 with the exception that segmentation was performed using Itlastik instead of Schnitzcells.

Segmentation was done either on the RFP (for Ptp-mCherry) or the YFP (for Pslm-mVenus) channel using the Itlastik pixel classification workflow. 64 Before import into Itlastik, fluorescent images were deconvolved applying the Lucy-Richardson method (as implemented in the Matlab ‘deconvfrcn’ function) using the experimentally determined point spread function (PSF) of the microscope. Pixels were then classified into two classes (‘background’ and ‘cells’), and the resulting probability images were imported into Matlab for post-processing. The cell class probabilities were smoothed using a Gaussian kernel (with a size of 1 pixel) and thresholded using a fixed threshold value of 0.6 to obtain putative cell masks. Subsequently, a binary mask was used to filter out sub-pixel holes in the cell masks, and a morphological opening operation (erosion followed by dilation) to separate adjacent cells. The morphological opening was done in two passes: First, all cell masks were opened by 1 pixel; subsequently, any remaining objects exceeding the expected cell wall thickness were attributed to potential cell clusters and a second opening by 2 pixels was applied to separate cells in these clusters. The resulting cell segmentation masks were then corrected using the Schnitzcells graphical user interface (GUI).

Cell tracking was performed with the automated tracking routine of Schnitzcells 1.3 (original version) 63. Subsequently, all tracking results were manually checked and corrected using the Schnitzcells GUI.

Cell features (length, growth rate, and mean fluorescence intensity as a proxy for gene expression level etc.) were extracted using a custom-written Matlab programme which had been previously developed for E. coli microcolony 65 and which was here adapted for S. meliloti. We summarize the most important details below.

Cell lengths were estimated using the method developed by Kiviet et al. 64 Here, a third-degree polynomial, f(x), is fitted to the cell mask. This polynomial is extrapolated by 10 pixels in both directions and the locations of the cell poles are determined automatically by calculating the silhouette proximity (sum of the distances between the poles to closest 25% of the cell mask) along the x axis of the silhouette. To detect the latter, this measure increases sharply at the cell poles, and the location of the poles can thus be taken as the points where the silhouette proximity reaches 110% of the average value in the cell centre. Subsequently, the cell length is calculated as 
\[
L = \frac{\int_{x_1}^{x_2} f(x) \, dx}{\int_{x_1}^{x_2} f(x) \, dx + x_1 \cdot x_2}
\]
where f(x) is the derivative of f(x) and x1 and x2 are the positions of the cell pole (x is the coordinate along the cell-centerline). In addition, the estimated cell lengths using the length of the major axis of an ellipse fitted to the cell masks (calculated using the Matlab ‘regionprops’ function). Overall, the two methods agree well, however, the first (based on polynomial fitting) is more robust to curved cell walls and it was therefore used for all data shown in the figures.

Cell growth rates, r, were calculated by fitting an exponential curve to time-traces of the measured cell length over time: 
\[
L(t) = L(0) \cdot e^{rt}
\]
To estimate the growth rate directly before and after cell division, we first estimated cell length measurement across divisions by summing up the cell lengths of the two daughter cells (extension after cell divisions) and by taking a fraction of \(L_1/(L_1+L_2)\), where \(L_1\) and \(L_2\) are the lengths of a cell and its sister at their birth (extension before cell is born). We then performed a linear regression on the log-transformed cell length over a sliding window of 11 time points (200 min) to obtain an estimate of the growth rate.

To accurately estimate expression levels of genes of interest, the respective fluorescence images were corrected for imaging artefacts, following the procedure described in van Vliet et al. 65. First, we performed a shading correction to correct for large image features of the NIS Elements images, as colonies then grew larger than the field of vision of the camera. In this case, stitching of the image stacks was performed immediately on phase-contrast images (15% overlap) using the NIS Elements software. Over the whole time-lapse experiment, focus was maintained using the Perfect Focus System (PFS). Furthermore, to facilitate focus maintenance, microscope and incubation chamber were preheated for at least 4–5 h, preferably even overnight.

Fluorophores were excited with lasers: mCherrulan with a 445 nm CUBE Laser (Coherent Inc., USA) [excitation band pass (ex) 440/45 nm, beam splitter (bs) 650 nm, emission band pass (em) 520/76 nm, em 580 nm] and mCherry with a 561 nm Sapphire Laser (Coherent Inc., USA) (ex bp 562/40 nm, bs 593 nm, em bp 624/40 nm). Laser intensities, exposure times and EM gains were applied as follows: 3%, 600 ms, 100 for Pslm-mVenus; 5%, 600 ms, 100 for Ptp-mCherry; 8%, 1, 100 for Pslm-mCherry; 4%, 1, 100 for Pslm-mVenus; 25%, 1, 5 s and 100 for Pslm-mCherry. For the 2×2 images, settings were modified as follows: 0.5%, 2×2 binning, 1, 100 for Pslm-mVenus; 0.5% 2×2 binning, 600 ms, 150 for Psoy-mCherrulan. Conversion gain was always set to 1. Generally, excitation intensities and exposure times were chosen as low as possible to minimize phototoxicity.

Processing, segmentation, tracking and single-cell analysis of early (2D) time-lapse data. The NIS Elements software was used to crop image stacks to the maximum spatial extent of the colony and to the time period during which cells were growing in a single layer. Further processing was done with a combination of Schnitzcells version 1.1 63, Itlastik version 1.3.3.post134, and a custom-built Matlab (MathWorks, Natick, Massachusetts) programme. 65 The workflow closely follows the pipeline developed by van Vliet et al. 7 with the exception that segmentation was performed using Itlastik instead of Schnitzcells.

Segmentation was done either on the RFP (for Ptp-mCherry) or the YFP (for Pslm-mVenus) channel using the Itlastik pixel classification workflow. 64 Before
values from mother and daughter cells. We thus obtained an estimated value of $\gamma = 0.055 \pm 0.010/\text{min}$. We defined pulses as a transient increase in $P$. Since a pulse can last longer than a cell life time, or begin in a mother cell and continue in one or both of its daughters, we needed a method that is not affected by cell division events to detect them. To this end we first traced all cell lineages backward in time; for each cell present in the last frame of the image stack we thereby obtained an extended lineage that starts at frame 1 with a founder cell and ends at the last frame with the focal cell itself. It is important to note that these lineages are not statistically independent—cells that occur early in a colony are of course part of multiple lineages; however, we correct for this at a later stage by removing all multiple detections. For each lineage we then used a peak finding algorithm (implemented in the Matlab function ‘peakfinder’) to find all candidate pulses. As this ‘peakfinder’ function considers symmetric prominence—i.e., both increase and decrease—we, subsequently calculated for each candidate pulse the prominence backward in time: This corresponds to an increase in the gene expression rate relative to the lowest value obtained since the last pulse, or since the beginning of the movie, whichever comes first. Only pulses with a prominence backward in time of more than 6 h were included; this threshold value had been determined based on visual inspection of a large number of trajectories of the strain with the lowest pulse frequency (the $\sin R$ promoter mutant), and the same threshold was used for all strains and conditions. Finally, we removed all duplicate detections and characterized each pulse by its prominence (backward in time, i.e., the increase), its absolute height, and the time since the last pulse. The average pulsing frequency per unit time was calculated for each colony as $f_{\text{puls}} = \frac{N_{\text{pulse}}}{dt \times n_i}$, where $N_{\text{pulse}}$ is the total number of pulses that occurred in the colony, $dt$ is the time interval between frames, $n_i$ is the number of cells present at frame $i$, and the sum is over all $T$ frames in the movie. The denominator measures the total observation time, taking into account that the number of cells increases over the duration of the movie.

**Flow cytometry and flow cytometry data analysis.** Starter cultures were prepared as described above. One millilitre of final cultures was harvested by centrifugation (4000 x $g$, 5 min, 4°C), resuspended in an equal volume of ice-cold phosphate-buffered saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na$_2$HPO$_4$, 0.24 g/l KH$_2$PO$_4$, pH 7.2), diluted to a final OD$_{600}$ of 0.0125 in ice-cold PBS and kept on ice until assayed. Flow-cytometry activated cell analysis was carried out with a BD LSRFortessa SORP flow cytometer (BD Biosciences, Germany). mNeonGreen intensity was assessed employing a 488 nm laser [band pass filter (bp) 50/20 nm], mVenus intensity employing a 561 nm laser (bp 562/72 nm), and mScarlet-I intensity employing a 605 nm laser (bp 586/15 nm) lasers.

Flow cytometry data were collected with BD FACS diva 8.0.1 (BD) in FCS 3.0 file format, and data analysis was carried out with FlowJo 10.6.0 software (BD). Gating (Supplementary Fig. 1) was first performed on forward and side scatterers (FSC and SSC, respectively) to remove dead cells and debris (SSC-A over FSC-A) and to exclude debris or small particles (e.g., streptavidin). Cell populations were first analyzed using FlowJo Exchange DownSample plugin, the number of events per sample was reduced to 10,000 x $g$, 5 min, 4°C. Most of the supernatant was decanted, cells resuspended in residual medium, transferred to pre-cooled 2 ml tubes and again pelleted by centrifugation (10,000 x $g$, 5 min, 4°C). After removal of all supernatant cells were resuspended in 2x Laemmli loading dye containing a calculated OD$_{600}$ of 20 and lysed by incubation at 95°C for 20 min and repeated vortexing. Samples were stored at −20°C for western blot analysis.

### Microplate reader fluorescence and optical density measurements.

To assess the effect of different sinR expression pulse frequencies on quorum sensing response dynamics, for each of the five strains with different sinR expression levels (analogous to the strains described in Fig. 2d, but without the fluorescent gene fusion) five test tubes with modified MOPS-buffered medium containing 0.5 mM IPTG were inoculated to five different OD$_{600}$ and grown overnight. The next morning, 2 ml of each culture were harvested and cells pelleted by centrifugation (4000 x $g$, 5 min, RT). Supernatants were transferred to fresh tubes, sterile-filtered, and 500 μl of sterile supernatants mixed with 500 μl of indicator strain culture adjusted to an OD$_{600}$ of 0.375. Each of the 25 supernatant-indicator strain suspensions, 3 x 100 μl were distributed in a 96-well microtiter plate as technical replicates. Further wells were filled with 3 x 100 μl of indicator strain mixed 1:2 with fresh medium, and with medium only as sterile/blank control. Plates were covered and incubated for 12 h in 5% CO$_2$ and 0.1% of the medium was removed by gentle pipetting.

For agarose pads, 1% (w/v) low melting agarose (Merck Sigma-Alrich) in EZRDIM was incubated at 70°C for 12 min to melt the agarose and then cooled down to 37°C. The agarose solution at 37°C was placed on an inverted microscope (Thermo Fisher, Germany), sealed with wax, and kept at -2°C until use. For each field of view a bright light snapshot was recorded to manually determine the number of cells.

### In vivo protein stability assay.

Starter cultures were prepared as described above. Prior to the experiment, 9 x 15 ml of 150 ml overnight culture were distributed in 100 ml Eppendorf tubes equilibrated to 30°C and further incubated for 15–30°C and shaking at 200 rpm. Chloramphenicol was added to the flasks at 20 μg/ml in 1-min intervals; after addition of chloramphenicol to the last flask, all flasks were shaken for another minute to ensure homogeneous distribution and uptake of the antibiotic even in the last sample. For harvest, all flasks were put on ice, 10 ml per sample was transferred to pre-cooled 1.5 ml tubes, and cells pelleted by centrifugation (10,000 x $g$, 5 min, 4°C). Most of the supernatant was decanted, cells resuspended in residual medium, transferred to pre-cooled 2 ml tubes and again pelleted by centrifugation (10,000 x $g$, 5 min, 4°C). After removal of all supernatant cells were resuspended in 2x Laemmli loading dye containing a calculated OD$_{600}$ of 20 and lysed by incubation at 95°C for 20 min and repeated vortexing. Samples were stored at −20°C for western blot analysis.

### Western blot analysis.

For more comparison of strain/growth condition effects via western blot analysis, cultures were prepared and harvested as above. Five microlitres of samples were loaded on a 12.5% SDS-polyacrylamide gel, and after electrophoresis separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Scientific) equilibrated in transfer buffer (0.025 M Tris, 0.192 M glycine, 20% (v/v) methanol) using the semi-dry blotting procedure. The membrane was then incubated with 1x phosphate-buffered saline supplemented with Tween-20 (PBST) (8 g/l NaCl, 1.44 g/l Na$_2$HPO$_4$, 2.0 g/l KCl, 0.24 g/l KH$_2$PO$_4$, 1 ml Tween-20, pH 7.2) containing 2% (v/v) methanol, 1 h at room temperature. The membrane was blocked with 5% (w/v) nonfat milk powder (blocking solution) and incubated with various antibodies. To assess an effect of different sinR expression levels on growth, starter cultures of the respective strains were diluted to an OD$_{600}$ of 0.15 in modified MOPS-buffered medium containing 0.5 mM IPTG, and 100 μl per well were distributed in a 96-well microtiter plate as technical replicates; further wells were filled with medium only as sterile/blank control. Plates were covered and incubated for 20 h in the same Infinite M Plex microplate reader set to 30°C and shaking at 200 rpm, and OD$_{600}$ was measured every 30 min using i-control 1.8 SP1 (Lifescience Tecan).

**Single-molecule microscopy, image processing and analysis.** Starter cultures were prepared as described above. Final cultures were harvested by centrifugation (4000 x $g$, 5 min, RT) and washed twice with 1 ml modified MOPS-buffered medium. Formaldehyde was then added to a final concentration of 3.7% (v/v), mixed gently by inversion and incubated for 15–20 min. After fixation, cells were washed once with 1 ml EZ rich medium (EZRDIM; Teknova, USA) and finally resuspended in 1 ml EZRDIM.

For agarose pads, 1% (w/v) low melting agarose (Merck Sigma-Alrich, Germany) in EZRDIM was incubated at 70°C for 12 min to melt the agarose and then cooled down to 37°C. The agarose solution at 37°C was placed on inverted microscope (Thermo Fisher, Germany), sealed with wax, and kept at -2°C until use. For each field of view a bright light snapshot was recorded to manually determine the number of cells.

For data analysis, each single-molecule movie was flattened to a single frame where each pixel was averaged over the entire movie. The resulting image was post-processed with the Thunderstorm Image plugin (https://github.com/citzen/thunderstorm) to count fluorescent spots. Here, an intensity threshold of 20 photons (based on the negative control strain lacking mScarlet-I) was used to avoid false-positive spots, and results were furthermore filtered in order to discard events outside of cells.
Protein production and purification. His6-ExpR was produced from pET28a-
expR, His6-GB1-SinR from pEM-GB1-sinR, and His6-GB1 from the empty pEM-
GB1 vector. E. coli BL21 (DE3) cells carrying the respective plasmids were grown at 37 °C under rigorous shaking in LB medium supplemented with 50 mg/l
kanamycin (for pET28a-expR) or 100 mg/l ampicillin (for pEM-GB1-sinR or pEM-GB1). At an OD600 of ~0.6, the culture was shifted to 20 °C and protein production was induced by addition of 1 mM IPTG. After further incubation for 20 h, cells were harvested by centrifugation (4000 × g, 20 min, 4 °C), resuspended in lysis buffer (20 mM of HEPES-Na pH 8.0, 20 mM KCl, 20 mM MgCl2, 250 mM NaCl and 40 mM imidazole) and lysed with an LM10 Microfluidizer (Micro-
fluidics) at 12,000 psi pressure. Cell debris was removed by centrifugation (47,850 × g, 30 min, 4 °C), and purification was then continued at room tem-
perature. The clear supernatant was loaded on a 1-mL HisTrap column (GE
Healthcare) equilibrated with 10 column volumes (CV) lysis buffer. After washing with further 10 CV of lysis buffer, proteins were eluted with 5 CV elution buffer (lysis buffer containing 500 mM imidazole). Proteins were further purified by size-exclusion chromatography (SEC) on a HiLoad 26/600 Superdex 200 pg
column (GE Healthcare) equilibrated with SEC buffer (20 mM of HEPES-Na pH 7.5, 20 mM KCl, 20 mM MgCl2, 200 mM NaCl). Fractions containing the desired protein were pooled, concentrated [Amicon Ultra-0.5 Centrifugal Filter Unit,
10 kDa MWCO (Millipore)], deep-frozen in liquid nitrogen and stored at –80 °C. Protein concentration was determined using a spectrophotometer (NanoDrop
Lite, Thermo Scientific).

Electrophoretic mobility shift assay (EMSA). A 177 bp Cy3-labelled fragment of the sinI promoter including the ExpR and SinR binding sites was generated via PCR with primers [Cy3]264 f and 440r. DNA fragments were mixed at 2.75 nM with purified proteins in reaction buffer containing 20 mM of HEPES-Na pH 7.5, 200 mM NaCl, 50 mM KCl, 20 mM MgCl2, 1 μg/μl bovine serum albumin (Sagga), 0.0025 μg/ml sonicated sperm DNA (GE Healthcare), 10 μM 3-oxo-C16:1-HSL (N-3-oxo-hexadec-11(Z)-enyl-1-homoserine lactone, Cayman Chemical), and 0.1% (v/v) DMSO in a final volume of 10 μl. If included, His6-ExpR was added at 1 μM, His6-GB1-SinR and His6-GB1 at 70 μM. Reactions were shielded from light and incubated for 30 min at room temperature. Subsequently, 2.5 μl loading buffer [5 parts 5× TBE buffer (Tris 54 g/l, boric acid 27.5 g/l, EDTA 0.0025 M, pH 8.3) mixed with 3 parts 87% glycerol] were added, and reactions were incubated for 30 min at room temperature. After electrophoresis (90 V, 2.5 h, covered from light), gels were scanned using a Typhoon imager (Typhoon Trio, Amersham Biosciences) and Typhoon Scanner Control v5.0 (GE Healthcare).

Statistical analysis, correlations and regressions. All statistical analysis, except for determination of means and medians of fluorescence intensities measured by flow cytometry (which were calculated by FlowJo), was performed with Graphpad Prism software (San Diego, California). To assess statistical significance of single-
molecule microscopy, time-lapse microscopy and flow cytometry data sets comparing two strains or growth conditions, two-tailed unpaired t-tests with Welch’s correction were performed, i.e., assuming that both groups of data were drawn from populations that at least approximately follow a Gaussian
distribution.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Pulse data are available in combination with the code, see below. Source data are provided with this paper.

Code availability
The custom code used for pulse analysis together with the respective data and instructions is available in the folder ‘Supplementary Data_Custom code’.

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Author contributions

V.B. and A.B.e conceived the study; V.B., B.T., M.M., W.S. and U.E. designed experiments; V.B., B.T., A.B.a and W.S. performed experiments; V.B. and B.T. analysed data; S.v.v. developed pulse analysis procedure and software; H.W. constructed and characterized the paco2 mutant; U.E. and A.B.e supervised work; M.M., U.E. and A.B.e acquired funding; and V.B. wrote the paper with input from all authors.

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Competing interests

The authors declare no competing interests.
