A segmentation clock patterns cellular differentiation in a bacterial biofilm

Kwang-Tao Chou\textsuperscript{1,6}, Dong-yeon D. Lee\textsuperscript{2,6}, Jian-geng Chiou\textsuperscript{1,6}, Leticia Galera-Laporta\textsuperscript{1}, San Ly\textsuperscript{1}, Jordi Garcia-Ojalvo\textsuperscript{3,7}, Gürol M. Süel\textsuperscript{1,4,5,7,8,*}

\textsuperscript{1}Molecular Biology Section, Division of Biological Sciences, University of California San Diego, La Jolla, CA 92093, USA

\textsuperscript{2}Department of Bioengineering and ChEM-H, Stanford University, Stanford, CA 94305, USA

\textsuperscript{3}Department of Experimental and Health Sciences, Universitat Pompeu Fabra, 08003 Barcelona, Spain

\textsuperscript{4}San Diego Center for Systems Biology, University of California San Diego, La Jolla, CA 92093-0380, USA

\textsuperscript{5}Center for Microbiome Innovation, University of California San Diego, La Jolla, CA 92093-0380, USA

\textsuperscript{6}These authors contributed equally

\textsuperscript{7}Senior author

\textsuperscript{8}Lead contact

Summary

Contrary to multicellular organisms that display segmentation during development, communities of unicellular organisms are believed to be devoid of such sophisticated patterning. Unexpectedly, we find that gene expression underlying the nitrogen stress response of a developing \textit{Bacillus subtilis} biofilm becomes organized into a ring-like pattern. Mathematical modeling and genetic probing of the underlying circuit indicate that this patterning is generated by a clock-and-wavefront mechanism, similar to that driving vertebrate somitogenesis. We experimentally validated this hypothesis by showing that predicted nutrient conditions can even lead to multiple concentric rings, resembling segments. We additionally confirmed that this patterning mechanism
is driven by cell-autonomous oscillations. Importantly, we show that the clock and wavefront process also spatially patterns sporulation within the biofilm. Together, these findings reveal a biofilm segmentation clock that organizes cellular differentiation in space and time, thereby challenging the paradigm that such patterning mechanisms are exclusive to plant and animal development.

**Graphical Abstract**

Bacterial biofilms exhibit a clock and wave-front process that spatially patterns sporulation without requiring long-range diffusion of molecular signals and is reminiscent of patterning mechanisms previously thought exclusive to plants and animals.

**Keywords**

Clock and wavefront; segmentation clock; somitogenesis; *Bacillus subtilis*; biofilm; nitrogen stress response; sporulation; pattern formation; multicellularity

**Introduction**

Multicellular organisms are well-known for displaying complex patterning of cell types during their development. For example, the formation of somites in vertebrates (somitogenesis) is primarily driven by cell-autonomous oscillations that arise from genetic
circuits (Henry et al., 2002; Holley et al., 2000; Hubaud et al., 2017; Masamizu et al., 2006; Oates and Ho, 2002; Palmeirim et al., 1997; Sawada et al., 2000; Tsiairis and Aulehla, 2016; Webb et al., 2016). As the organism grows, these oscillations freeze in place and create spatially repeating patterns of cellular differentiation. In this way, temporal oscillations are converted into spatially repeating patterns. This mechanism of spatial patterning during development is known as a segmentation clock, or a clock and wavefront process (Aulehla et al., 2008; Cooke and Zeeman, 1976; Oates et al., 2012; Pourquie, 2003). Such a patterning mechanism can give rise to repeating structures (segments) that determine somite boundaries. To date, this type of mechanism has only been observed in vertebrates, arthropods, and plants (Richmond and Oates, 2012), and it is thus assumed that such sophisticated patterning mechanisms are not present in “simple” bacterial systems.

Bacterial biofilms are large cellular communities encapsulated in an extracellular matrix, which contain cells in distinct physiological and morphological states, including spores. The spatial organization of cell types in biofilms is commonly believed to follow monotonic gradients such as nutrient, or oxygen availability (Nadezhdin et al., 2020; Srinivasan et al., 2018). For example, nitrogen sources are expected to be readily available at the periphery of the biofilm and assumed to be limited towards the interior of the biofilm (Figure 1A) (Billings et al., 2015; Liu et al., 2015; Prindle et al., 2015; Stewart and Franklin, 2008). Since nitrogen limitation can trigger sporulation (Dawes and Mandelstam, 1970), the spatial distribution of spores within the biofilm could be expected to reflect such simple nutrient gradients and therefore be primarily localized at the center of the biofilm. However, since bacteria respond to starvation by inducing the expression of specific genes to mitigate the stress (Irving et al., 2021; Volker et al., 1999; Wang and Levin, 2009), the relationship between nutrient gradients and the distribution of cell states might not be straightforward. It thus remains unclear whether bacterial activity in biofilms can generate more complex spatial distributions of cellular fates.

Here we uncovered a clock and wavefront mechanism that patterns nitrogen stress response and subsequently sporulation during the development of undomesticated bacterial *Bacillus subtilis* biofilms. We identified the genetic circuit underlying oscillations in the nitrogen stress response that generates the community-level concentric rings of gene expression patterns. Furthermore, we show that these response dynamics result in the patterning (segmentation) of sporulation in space and time during biofilm development. In this way, biofilms can utilize a clock and wavefront mechanism similar to somitogenesis to organize cell types within the biofilm. Our discovery demonstrates that bacterial biofilms employ a developmental patterning mechanism hitherto believed to be exclusive to vertebrate and plant systems.

## Results

### Nitrogen Stress Response in the Biofilm is Organized into a Ring-Like Pattern

We began by measuring the spatial profile of the *B. subtilis* stress response, to determine whether it is consistent with the expected monotonic nutrient gradient within a developing biofilm (Figure 1A). To measure the nitrogen stress response, we grew a biofilm (Figure 1B, C) on the surface of an agar pad containing defined minimal media (MSgg). Specifically,
we monitored a genetically encoded fluorescent reporter (YFP) for the promoter activity of the nasA gene using time-lapse imaging (Figures 1D and 1E). The B. subtilis nasA gene encodes a nitrate transporter that is upregulated when nitrogen is limited (Nakano et al., 1995). The expression of the nasA promoter thus directly reports the native cellular nitrogen stress response. Counterintuitively, the pattern of nasA expression did not follow a monotonic profile, but rather manifested a ring-like pattern within the biofilm that arises after approximately 20 hours of growth (Figures 1E, 1G and S1).

To exclude potentially trivial explanations of this unexpected nitrogen response pattern, such as differences in cell density and biofilm 3D organization, we constructed a dual-reporter strain that, in addition to PnasA-yfp, also contained a CFP reporter expressed from the IPTG-inducible hyperspank promoter (Figure 1D). At full induction (1 mM IPTG), the expression of the hyperspank promoter is constitutive and should report any cell density and 3D structural features of the biofilm. In contrast to nasA expression, we find that Phyperspank expression exhibits a simple spatial profile (Figures 1F, 1G and S1), with the fluorescence signal being high at the center and then gradually decreasing towards the edge of the biofilm. The ring-like pattern of the nitrogen stress response therefore cannot be simply explained by differences in cell density or 3D features of the biofilm. The observed ring-like pattern in nitrogen stress response during biofilm development thus appears to be generated by a regulated process.

**A Clock and Wavefront-Based Mathematical Model Predicts the Ring-Like Pattern**

To identify the mechanism that could be responsible for the observed ring-like gene expression pattern, we focused on the underlying nitrogen stress response circuit (Figure 2A, see S2A for a detailed circuit diagram). The nitrogen source (glutamine) binds the enzyme glutamine synthetase (GS), thereby forming the complex known as feedback-inhibited GS (FBI-GS) (Deuel and Prusiner, 1974). This FBI-GS complex inhibits the activity of the transcriptional regulator TnrA (Wray et al., 2001), which in turn regulates various downstream genes related to nitrogen-stress (Gunka and Commichau, 2012; Mirouze et al., 2015; Sonenshein, 2007). The inhibition of TnrA activity by FBI-GS is released under nitrogen stress, as glutamine limitation leads to a decrease in FBI-GS. The resulting higher TnrA activity promotes higher glutamine levels in cells through two distinct ways: First, it activates various nitrogen transporter genes including the nitrate importer gene nasA (Nakano et al., 1995). Second, it inhibits the gltAB operon (Belitsky et al., 2000), which encodes glutamine oxoglutarate aminotransferase (GOGAT) that consumes glutamine. Once glutamine becomes abundant again, TnrA inhibition through FBI-GS is re-established.

Collectively, these interactions comprise a negative feedback loop that regulates the level of intracellular glutamine (Figure 2A). We modeled this negative feedback loop acting on glutamine as a set of ordinary differential equations (ODEs) based on mass-action kinetics of the underlying chemical reactions (Figure S2A, see Methods for details). Our modeling results reveal that the genetic circuit can generate a temporal pulse in the nitrogen stress response (Figure 2B).

How can a system convert a temporal pulse into a spatial pattern? A well-known process by which temporal dynamics can be translated into spatial patterns is the clock and wavefront
mechanism, which can transduce oscillations into spatially repeating patterns (Oates et al., 2012; Pourquie, 2003). The clock and wavefront mechanism consists of two necessary components: (i) a cell-autonomous oscillator (the clock), achieved by a negative feedback loop like the one described above (Figures 2A and 2B), and (ii) a propagating wavefront that allows the state of the cells to be “frozen” at a particular phase of the oscillation. In an expanding biofilm, this “freezing” mechanism might naturally occur during development: Replicating cells at the leading edge of biofilm grow, leaving behind daughter cells that become embedded within the biofilm and thus have less nutrient access. Reduced nutrient availability could result in cells “freezing” in the last state of their metabolic activity (Liu et al., 2015). Given that these two features can be found in growing biofilms, it may be possible for a clock and wavefront model to account for the observed spatial ring-like pattern that forms during biofilm development.

Using mathematical modeling, we investigated whether the clock and wavefront mechanism described above is sufficient to generate a ring-like spatial pattern in biofilms. We built an agent-based model with individual cells occupying a grid on a one-dimensional radial axis (Figures 2C and S2B). We integrated the ODEs of the glutamine negative feedback loop with a space-dependent metabolic “freeze term”. The overall reaction rate in each cell was factored by this freeze term, which slows the reaction down as the distance from the biofilm edge increases (see Methods for details). The clock therefore runs at full speed in cells at the edge of the biofilm (“clock cells”) and gradually slows down below the wavefront for cells in the interior (Figure 2C). We find that in this way the clock and wavefront model can preserve the temporal pulse of the simulated nasA expression and generate a spatial ring-like pattern as the system expands (Figures 2D and S2C). The clock and wavefront mechanism is thus sufficient to explain the formation of the experimentally observed ring-like nitrogen stress response pattern (Figures 1E and 1G).

Biofilms Can Produce Multiple Concentric Rings of Nitrogen Stress Response

To pursue a stringent test of the clock and wavefront model, we asked whether a developing biofilm can even generate multiple concentric rings. If the negative feedback oscillates in multiple cycles within the time frame of biofilm development (~48 hours), the clock and wavefront mechanism could translate the oscillations into multiple concentric rings, similar to segments in vertebrates. Given that biofilm growth ceases after about 2 days, we investigated whether it would be possible to speed up the metabolic dynamics so that several cycles could fit within this time window (Figure 3A). We aimed to decrease the period of oscillations without genetically manipulating the undomesticated biofilms. Specifically, we investigated whether the oscillation period could be sped up by simply changing the concentrations of the nitrogen source (glutamate), or the major carbon source (glycerol) (Figure 3B). We modeled a change in glutamate concentration as a change in glutamate supply rate, which in turn changes glutamine synthesis (see Methods for details). We modeled a change in glycerol concentration as a change in 2-oxoglutarate synthesis rate, since 2-oxoglutarate connects carbon to nitrogen metabolism (Gunka and Commichau, 2012; Sonenshein, 2007). Our results show that higher glutamate concentration shortens the period due to a decrease in the effective time-delay of the negative feedback (Figure S3A). This emergent effect is similar to traditional results obtained where time delay.
is considered explicitly (Lewis, 2003). In contrast to the effect of increased glutamate, higher glycerol concentration does not markedly alter the period, but rather contributes to increasing the parameter range over which oscillation occurs (Figure S3B). In the two-dimensional parameter space of glycerol and glutamate modulation (Figures 3C), we find that proportionally increasing both glutamate and glycerol concentrations (Figure 3C, black arrow) leads to oscillations that exhibit the desired shorter period (Figure 3D) over a broad range of parameter values (Figure 3E). The agent-based model thus predicts the emergence of a double-ring pattern (two concentric rings) in the biofilm under combined higher glutamate and glycerol concentrations (Figures 3F and 3G). According to our model, it may be experimentally feasible to obtain multiple rings of gene expression during biofilm development if glutamate and glycerol concentrations are both proportionally increased.

We experimentally tested the important modeling prediction that a double-ring pattern of gene expression can emerge in a developing biofilm. In accordance with our modeling results, we increased by three-fold the concentration of both glutamate and glycerol in the MSgg growth medium. We indeed find that biofilms grown under these nutrient conditions generate two temporal pulses of nasA expression, resulting in two concentric rings of gene expression (Figures 3H–3K, Movie S1). In fact, we could even detect the onset of a third ring, but the biofilm growth ceased before it become prominent (Figure S4, Movie S2). Importantly, we again emphasize that these multiple rings were achieved without any genetic or drug-based modifications. These results demonstrate that the nitrogen stress response of biofilms is intrinsically able to generate multiple spatio-temporal oscillations of nasA expression that results in a spatial pattern of concentric rings, thereby strongly supporting our clock and wavefront model.

Validation of the Clock Components

To further validate the clock underlying our model, we specifically tested the role of negative feedback loop components (Figure 4A). We first made two point mutations in the TnrA binding site of the nasA promoter containing the YFP reporter (P_{nasA}(mut), see Methods for details) (Nakano et al., 1995). As expected, we no longer observed the ring-like gene expression pattern (Figure S5A–S5C), confirming that the P_{nasA}-yfp construct reports the activity of TnrA. Next, we asked if TnrA inhibition by glutamine is a critical aspect of the negative feedback loop. To test this, we constructed a point mutation that disrupts the FBI-GS binding site of TnrA (Wray and Fisher, 2007). Consistent with modeling simulations (Figure 4B), the mutant (TnrA{M96A}) biofilm exhibited sustained nasA expression at all timepoints and across the entire biofilm (Figure 4C). This indicates that regulation from FBI-GS is critical in turning off TnrA activity and subsequently nasA expression. To test the importance of the negative feedback via GOGAT, we deleted gltA. We find that in agreement with modeling simulations (Figure 4D), the gltA deletion strain disrupted the multiple ring pattern and exhibited low nasA expression over the entire biofilm (Figure 4E). This result shows that the GOGAT-mediated negative feedback loop is crucial for oscillations. Consistent with this mechanism, spatial expression of gltA in the biofilm is anti-correlated with nasA expression in the P_{gltA}-yfp, P_{nasA}-cfp dual reporter strain (Figures S5D–S5G). This anti-correlated expression of nasA and gltA not only further supports our model, but also clearly demonstrates that the observed complex spatial gene expression
patterns are actively generated by the nitrogen stress response circuit. Together, these results confirm the critical role of the negative feedback loop structure and its components in the generation of the non-trivial, ring-like *nasA* expression pattern.

**Validation of the Wavefront**

We next set out to identify and characterize experimentally the wavefront component of the system. According to the model, the wavefront propagates outwards as the biofilm grows, freezing the clock at specific phases of the oscillation and thereby generating the stationary ring pattern (Figure 2C). In our mathematical model, we define the wavefront as the boundary at which the metabolic activity (given by the “freeze term” described in the Methods section) has decayed to 50% of its maximum level (Figures 4F and 4G). Simulations show that *nasA* oscillations should occur only ahead of the wavefront, but not behind it (Figure 4H). To test this prediction experimentally, we first identified the wavefront by using changes in the expression of the transcriptional reporter *P*<sub>hyperspunik</sub>-<em>c</em>f<em>p</em> (Figures 4I, S5H and S5I) as a proxy for metabolic activity. We defined the wavefront as the boundary where the CFP signal difference between consecutive time points is at 50% of its maximum (which is the same threshold used in our simulations). We then averaged the *P*<sub>nasA</sub>-y<em>f</em>p signal difference separately for the regions ahead of and behind this wavefront (Figure 4J). In agreement with the model, *nasA* activity shows clear oscillations ahead of the wavefront, but not behind it. Together, these data show that, consistent with the clock and wavefront model, nitrogen-stress oscillations become “frozen” into a spatial pattern as the wavefront crosses cells.

**Oscillation of the Nitrogen Stress Response is Cell-Autonomous**

Next, we investigated whether the observed concentric ring pattern is generated by a cell-autonomous process, as predicted by our model. A fundamental property of the clock and wavefront mechanism is that cell-autonomous oscillations generate the spatial patterns without the requirement of long-range diffusion of signaling molecules. This property distinguishes the clock and wavefront mechanism from others, such as reaction-diffusion systems, which can also generate spatial patterns but require the diffusion of instructive signals (Basu et al., 2005; Cao et al., 2016; Cotterell et al., 2015; Meinhardt, 2008). To determine if long-range diffusion plays a key role in the observed concentric ring patterns, we inserted a diffusional barrier during biofilm development (Figure 5A). Specifically, after the biofilm generated its first ring of *nasA* expression, we inserted a physical barrier (plastic separator) between the ring and the biofilm periphery (first two panels in Figure 5B). This barrier penetrates all the way through the depth of the agar and blocks diffusion of chemicals between the separated regions of the biofilm (Figure S6). After insertion of the physical barrier, the biofilms continued to grow at the periphery and formed the expected second ring of *nasA* expression without spatial distortion (Figure 5B). This finding argues against mechanisms mediated by long-range diffusion of signals and suggests that the observed concentric-ring patterns are driven by cell-autonomous oscillations.

To more directly test whether individual cells indeed exhibit oscillations as predicted by the clock and wavefront model (Figure 5C), we tracked the nitrogen stress dynamics of individual cells (Figure 5D). Since tracking of individual cells within a large biofilm
is experimentally challenging, we opted for measuring the $\text{P}_{\text{nasA}}$ reporter dynamics in microcolonies, composed of hundreds of bacteria. To mimic the nitrogen limitation within the biofilm, we used media with 500-fold lower nitrogen (glutamate) concentration compared to the medium used for biofilm growth on agar. As predicted by our clock and wavefront model, we indeed observed oscillations of the $\text{nasA}$ promoter expression in single cells (Figures 5D and 5E). The period of $\text{nasA}$ oscillations in single cells was similar to the period observed in biofilms with concentric rings (approximately 15 hours, Figures 3H and 5E). The observed nitrogen stress response dynamics in biofilms thus appear to result from single cell-level oscillations, consistent with one of the key features of the clock and wavefront mechanism.

### Pulses of Nitrogen Stress Response Give Rise to Pulses of Sporulation

Nitrogen stress is known to trigger sporulation in *B. subtilis* (Dawes and Mandelstam, 1970; Schaeffer et al., 1965). Therefore, we tested the expectation that peaks in $\text{nasA}$ expression may precede sporulation. Imaging of microcolonies revealed that sporulation was visible by phase contrast optics (Figure 5D). To precisely examine the relationship between nitrogen stress response and sporulation, we constructed a dual-reporter strain ($\text{P}_{\text{nasA}}$-$\text{cfp}$, $\text{P}_{\text{spoIIR}}$-$\text{yfp}$). This strain allowed us to simultaneously monitor nitrogen stress response as well as irreversible commitment to sporulation, as reported by $\text{spoIIR}$ expression (Karow et al., 1995; Kuchina et al., 2011; Londono-Vallejo and Stragier, 1995). By simultaneously tracking the activities of these two promoters at the single-cell level in microcolonies, we found that sporulation follows the peaks in nitrogen stress oscillations with a time delay of approximately 5 hours (5.6 ± 3.5 SD) (Figures 6A–6C). Specifically, after each peak of $\text{nasA}$ expression, cells expressed $\text{spoIIR}$ and a subset of them sporulated, while the rest continued to oscillate in $\text{nasA}$ expression. Cells that did not sporulate after the first pulse of $\text{nasA}$ expression were observed to sporulate at subsequent $\text{nasA}$ pulses (Figure 6B–6D). These results confirm that sporulation is promoted by nitrogen stress and, more importantly, suggest that oscillation in the nitrogen stress response could spatially pattern sporulation in biofilms.

### Sporulation in Biofilms is Patterned by Nitrogen Stress Response

Based on our single-cell measurements, we hypothesized that sporulation follows the ring pattern of nitrogen stress in the biofilm. In other words, we expected to see a spatial correlation between the concentric rings of $\text{nasA}$ expression and regions of higher sporulation. To test this hypothesis, we monitored the spatiotemporal dynamics of the dual-reporter strain described above ($\text{P}_{\text{nasA}}$-$\text{cfp}$, $\text{P}_{\text{spoIIR}}$-$\text{yfp}$) in biofilms (Figures 6E and 6F). As expected, we found that sporulation occurs in a ring-like pattern similar to the nitrogen stress response pattern. The maximum intensities measured over time show that the spatial patterns of $\text{spoIIR}$ expression in concentric rings follows those of $\text{nasA}$ expression in the biofilm (Figures 6G–6I). Furthermore, the sporulation “segments” appeared with a time delay relative to the nitrogen stress response. This time delay in biofilms was on same order of magnitude as observed with single cell measurements (Figures 6A–6C and 6E–6F). Due to this time-delay, the $\text{nasA}$ and $\text{spoIIR}$ rings do not perfectly overlap in space (Figure 6I). The spatiotemporal concurrence of the nitrogen stress response and sporulation (Figure 6J)
suggests that spore formation within the biofilm is patterned by the clock and wavefront mechanism.

Discussion

Our results show that nitrogen stress response within a *B. subtilis* biofilm can inherently form a complex pattern of concentric rings. More specifically, as the biofilm expands by cell replication, temporal oscillation in nitrogen stress response appears to be imprinted into spatial concentric rings through a clock and wavefront mechanism. We further show that the temporal oscillations in nitrogen stress response are cell-autonomous and influence sporulation dynamics. Consequently, the observed concentric ring pattern is converted into a “fossil record” by sporulation. Single cell measurements in microcolonies also capture the corresponding global dynamics observed in large biofilms. A growing community of oscillating bacteria can thus generate spores in a spatially and temporally organized manner. In other words, the biofilm appears to utilize the oscillating nature of the nitrogen stress response as a means to pattern sporulation within a multicellular community.

Various patterning mechanisms have been identified in biological systems, provoking the question of whether the clock and wavefront mechanism may provide some benefit for biofilms. Firstly, biofilms consist of individual cells that are embedded in a dense extracellular matrix (Billings et al., 2015; Vlamakis et al., 2013). This organization may lead to physical barriers that make it difficult to drive biofilm-level coordination through long-range diffusion of signals. Therefore, mechanisms that rely, for example, on diffusion-based morphogen gradients as described in *Drosophila* embryos (St Johnston and Nusslein-Volhard, 1992) may not be ideal for patterning of biofilms. Secondly, the biofilm is a growing and spatially expanding system similar to vertebrate embryos. If a pattern were formed through a diffusion-based mechanism, it would migrate as the system expands and individual cells would receive inconsistent differentiation signals. In contrast, the clock and wavefront mechanism is based on a cell-autonomous clock driven by a genetic circuit that is likely more robust to changes in system size. The cellular independence of the clock and wavefront mechanism suggests a more reliable patterning of the biofilm.

While bacteria are notorious for utilizing stochastic cell-to-cell variability as a bet-hedging strategy (Balaban et al., 2004; Nadezhdin et al., 2020; Suel et al., 2006; Veening et al., 2008), our results indicate that development of bacterial biofilm communities is governed by a deterministic clock and wavefront process. In particular, biofilms appear to utilize an oscillatory process that can locally amplify nitrogen stress responses, such that spores may form even under more abundant nutrient supply closer to the biofilm edge. In this way, spores are not restricted to form only in the starved interior of the biofilm. The ring-like patterning mechanism described here allows the biofilm to form spores at different times and in distinct locations (segments) of the biofilm, without being strictly tied to nutrient availability. It is plausible to speculate that such spatiotemporal cell-type patterning may be beneficial to cope with unpredictable changes in future conditions, as not all spores are formed at the same time and the same region of the biofilm. The biofilm may thus employ a bet-hedging strategy typically associated with stochastic unicellular organisms, but using the deterministic patterning mechanism of multicellular organisms. Our work thus indicates
a paradigm for a bacterial system that blends unicellular and multicellular strategies for cellular differentiation.

We show here that the clock and wavefront mechanism established for vertebrate somitogenesis is surprisingly applicable to biofilm development. In both cases, the clock and wavefront generates patterns by “freezing” the different phases of an oscillation in the form of spatially repeating segments. Furthermore, we find that the pattern generated by the nitrogen stress response in turn defines spatial regions within the biofilm where cells differentiate into spores. Cellular differentiation within the biofilm thus becomes “segmented” similar to somitogenesis in developing embryos. We thus present a bacterial paradigm for developmental patterning with fewer biological complexities and experimental limitations compared with the study of vertebrate embryos. While there are undoubtably marked differences, our findings provoke a reconsideration of the commonly assumed divide between “simple” unicellular organisms and “complex” multicellular organisms such as vertebrates.

Limitations of the Study

While our work identifies an intriguing similarity between patterning of cellular differentiation during bacterial biofilm development and mammalian somitogenesis, we would like to point out that this similarity is at the conceptual level, and of course detailed molecular mechanisms are distinct. Furthermore, recent literature on vertebrate systems has proposed alternatives to the clock-and-wavefront mechanism (Lauschke et al., 2013; Murray et al., 2011; Pais-de-Azevedo et al., 2018; Shih et al., 2015; Soroldoni et al., 2014). Distinguishing between some of these mechanisms in a *B. subtilis* biofilm can be challenging due to the fundamental differences in detailed molecular mechanisms and components. For instance, *B. subtilis* biofilms do not exhibit a clear-cut boundary between cell types equivalent to the sharp transition between presomitic mesoderm and somites in vertebrates. We thus cannot conclusively determine whether alternatives to the clock-and-wavefront mechanism, such as those based on oscillator phase-gradient (Lauschke et al., 2013), could play a role in biofilm segmentation. In addition, here we use *B. subtilis* biofilms grown on agar pads, which allows us to experimentally study large biofilms (>1 cm across), but this in turn limits our access to single-cell resolution measurements in these large communities. We were therefore unable to directly track in such large biofilms the decline of oscillation in single cells as the wavefront passes, or determine whether autonomous oscillations in adjacent cells may become coupled. Future studies are required to elucidate such questions. In spite of these limitations, our findings are likely to inspire new studies to better understand not only the differences, but more importantly the intriguing commonalities of segmentation patterning among evolutionary distant developmental systems.

**STAR Methods**

**RESOURCE AVAILABILITY**

**Lead contact**—Further information and requests for resources and reagents can be directed to and will be fulfilled by the Lead Contact, Gürol M. Süel (gsuel@ucsd.edu).
Materials availability—This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- All original code has been deposited at Zenodo and is publicly available as of the date of publication. DOI is listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial strains—The Bacillus subtilis strains used in this study, as well as vectors and primers used to generate the strains, are listed in key resources table. Briefly, four kinds of chromosomal integration vectors are used to construct strains: (1) pSac-cm (ECE174: Bacillus Genetic Stock Center) is used for sacA locus integration, (2) pDL30 (kind gift from Jonathan Dworkin, Columbia University) is used for amyE locus integration, (3) pER449 vector is a generic integration vector used to introduce a mutation in the coding region of tnrA gene to generate TnrAM96A strain, and (4) ECE173 is used to generate the gltA deletion mutant. ECE174-PnasA-yfp, pDL30-PnasA-cfp, pDL30-PcitZ-cfp, pDL30-Psucc-cfp vectors are kind gifts from the Michael Elowitz lab (Caltech, CA). PnasA(mut)-yfp construct was generated based on PnasA-yfp using primers GS1871 and GS1872. It contains two point mutations in the well-characterized TnrA binding sequence (TGTCAAAAAAACTTACA → TATCGCAAAAAACTTACA). These mutations were characterized in Nakano et al. (1995). TnrAM96A mutant is constructed using primers GS 1865 – 1870 based on Wray and Fisher et. al. (2007).

METHOD DETAILS

Growth conditions—Biofilms were grown on MSgg plates: MSgg medium [5 mM potassium phosphate buffer (pH 7.0), 100 mM MOPS buffer (pH 7.0, adjusted with NaOH), 2 mM MgCl2, 700 mM CaCl2, 50 mM MnCl2, 100 mM FeCl3, 1 mM ZnCl2, 2 mM thiamine HCl, 0.5% (v/v) glycerol, 0.5% (w/v) monosodium glutamate] supplemented with 1.5% agar (w/v). Glutamate and iron stocks were made fresh for each experiment. MSgg medium was made fresh from stocks the day of the experiment. 3xMSgg medium used to create multiple concentric rings was the same as regular MSgg medium except three times the normal amount of glycerol and three times the normal amount of glutamate. MSgg medium used in single cell experiments (scMSgg) used 1.5% (w/v) glycerol and 0.003% (w/v) glutamate, which has 500 times less glutamate than the biofilm condition to mimic the nitrogen limitation within the biofilm. The Phyperspank reporter was induced using 1 mM IPTG.

Biofilm growth—The day before the experiment, MSgg or 3xMSgg plate was poured to a height of 2.5 mm and left to set for 5–10 minutes. The plate was then left upside-down at 30°C overnight. Desired strains were streaked out from ~80°C glycerol stocks onto separate LB plates with appropriate selection antibiotics. The following day, a single colony was picked from the plate and inoculated into 2 mL of LB broth. After 4.5 hr of shaking growth
at 37°C, the optical density (OD) was measured and normalized such that all strains had the same OD of 1.3 in 1.5 mL total volume. Cells were then spun down at 2100 rcf for 1.5 min and resuspended in 1.5 mL MSgg. They were then grown at 37°C with shaking for 1.5 hr for cells to acclimate to the MSgg media. Then, 1 μL of cell culture was spotted into the middle of each well of the 12-well plate and imaged.

**Single cell growth**—The day before the experiment, 15 mL scMSgg media supplemented with 1.5% (w/v) agar was poured into a petri dish of diameter 10 mm. After solidifying, the plate was then left upside-down at 30°C overnight. Desired strains were streaked out from −80°C glycerol stocks onto LB plates with appropriate selection antibiotics. The following day, a single colony was picked from the plate and inoculated into 2 mL of LB broth.

After 2.5 hr of shaking at 37°C, the optical density (OD) was measured. Cells were then normalized such that all strains had a final OD of 1.3 in 1 mL total volume and spun down at 2100 rcf for 1 min. Cells were resuspended in 1 mL MSgg and spun down again at 2100 rcf for 1 min. Pellets were resuspended in 0.5 mL MSgg and grown with shaking at 37°C for 1.5 hr to acclimate to the MSgg media. During this time, 1 cm by 1 cm pads were cut out from the plate made the day before. Then, 2 μL of cell culture was spotted into the middle of each pad, each pad flipped upside-down onto a glass-bottom dish, and imaged.

**Time-lapse microscopy**—Biofilm dynamics were observed via time-lapse microscopy using an Olympus IX81 microscope with a Lambda XL light source from Sutter Instruments and a 2.5x objective. Images were taken with an ORCA-Flash4.0 V2 camera (Hamamatsu) at 40-minute intervals. Single cell dynamics were observed via time-lapse microscopy using an Olympus IX83 microscope with an X-Cite LED light source from Lumen Dynamics and a 100x objective. Images were taken at 20-minute intervals.

**Mathematical model**—Our clock and wavefront model considers a molecular clock that oscillates in each individual cell in an expanding biofilm. We used nine ordinary differential equations (ODEs) to describe the mass-action reactions between each component i of glutamine metabolism that drives and modulates the molecular clock (Figure S2A, Figure 2A)).

\[
\frac{dE}{dt} = aE - aNE + \beta_{QAOQ} - \gamma_{QSE}E
\]

\[
\frac{dN}{dt} = aNE - aQSEN
\]

\[
\frac{dO}{dt} = aO + aNE - \beta_{QAOQ} - \gamma_{QO}
\]

\[
\frac{dS}{dt} = aS - \beta_{SS} - \frac{aF^m}{K_F + Q^m}
\]
\[
\frac{dQ}{dt} = a_Q S E N - \beta_Q AOQ - \gamma_Q Q \\
\frac{dF}{dt} = \alpha_F S Q^m \frac{Q^m}{K_F^m + Q^m} - \beta_F F \\
\frac{dT}{dt} = \alpha_T (T - T) - \beta_T FT \\
\frac{dA}{dt} = \alpha_A \frac{K_T^A}{K_T + T} - \beta_A A \\
\frac{dR}{dt} = \alpha_R \frac{T^n}{K_R + T^n} - \beta_R R - \gamma R 
\]

The cycle between glutamate (E) and glutamine (Q) lies at the intersection between nitrogen and carbon metabolism. Glutamate is provided externally from the media as the only nitrogen source to the system. GS (glutamine synthetase, S) catalyzes the conversion of glutamate and ammonium (N) to form glutamine (Q). We assume that ammonium is exclusively provided by the degradation of glutamate at a constant rate. GOGAT (glutamine oxoglutarate aminotransferase, A) in turn converts glutamine into glutamate by transferring an amino group to 2-oxoglutarate (O). 2-oxoglutarate is provided externally from glycerol through the TCA cycle or from degradation of glutamate. The GS-GOGAT enzymatic reactions are modeled by mass-action kinetics assuming that substrate concentrations are below the capacity of the enzymes.

The negative feedback loop that regulates glutamine (Q) via FBI-GS (F), TnrA (T), and GOGAT (A) underlies the core oscillator (Figures 2A and S2A). A minor portion of glutamine (neglected in the glutamine equation) and its downstream nitrogen-containing metabolites bind to GS to form FBI-GS (feedback-inhibited GS, F). The cumulative nature of GS inhibition was modeled with a Hill function. FBI-GS binds and sequesters the transcription factor TnrA, which we model as a simple binding-unbinding process with the total amount of TnrA assumed constant. Free TnrA (T) indirectly inhibits GOGAT and enhances the production of the fluorescent reporter PnasA-yfp (R), both of which we also model with Hill functions. In addition to protein degradation, we also assume minor photobleaching of the fluorescent reporter R.

We integrate the metabolic reactions into individual cells in an expanding biofilm. The expansion of the biofilm is modeled as an array of single cells in one spatial dimension along the radial axis (r) of the biofilm (Figures S2B–S2C). We modeled the expansion in
a simplified scenario by assuming duplication at only periphery-most cell, while the rest of the cells remains in place (Figures S2B–S2C). As the cell at the periphery continues to duplicate, cells that were born earlier become positioned at the interior of the biofilm with increasing distances from the periphery of the biofilm. To account for the decrease in nutrient availability with increasing distance to the periphery, we define a metabolic freeze term \( g(r,t) \) as:

\[
g(r,t) = \frac{e}{1 + \eta \cdot (r_0 + k t - r)}
\]

where \( r \) is the position of a given cell, \( e \) is a factor that scales the metabolic rates to match the two-dimensional growth of biofilms in experimental data, \( r_0 \) marks the edge of the area where the initial cell culture was plated to grow a biofilm, \( k \) denotes the expansion rate of the biofilm, and \( \eta \) is a multiplicative factor that relates the distance (and thus the nutrient availability) to the decrease in metabolic activity. We consider that this freeze term acts multiplicatively on all reaction terms of the differential equations given above, except on the photobleaching of the fluorescent reporter R.

The differential equations given above are solved by MATLAB (MathWorks Inc.) solver ode45. Cell duplication was treated as a discrete process. The parameter values are listed as follows, unless otherwise specified in figure legends:

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Description</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha_E )</td>
<td>Glutamate supply rate</td>
<td>2</td>
<td>mM·h(^{-1})</td>
</tr>
<tr>
<td>( \gamma_E )</td>
<td>Glutamate degradation rate by other metabolic processes</td>
<td>0.1</td>
<td>h(^{-1})</td>
</tr>
<tr>
<td>( \alpha_N )</td>
<td>Ammonium production rate</td>
<td>0.1</td>
<td>h(^{-1})</td>
</tr>
<tr>
<td>( \alpha_Q )</td>
<td>Glutamine synthetase (GS) reaction rate</td>
<td>10</td>
<td>mM(^{-2})·h(^{-1})</td>
</tr>
<tr>
<td>( \beta_Q )</td>
<td>GOGAT reaction rate</td>
<td>(10^3)</td>
<td>mM(^{-2})·h(^{-1})</td>
</tr>
<tr>
<td>( \alpha_O )</td>
<td>2-oxoglutarate supply rate from the TCA cycle</td>
<td>10</td>
<td>mM·h(^{-1})</td>
</tr>
<tr>
<td>( \gamma_O )</td>
<td>2-oxoglutarate degradation rate through the TCA cycle</td>
<td>10</td>
<td>h(^{-1})</td>
</tr>
<tr>
<td>( \alpha_S )</td>
<td>Glutamate synthetase production rate</td>
<td>10</td>
<td>(\mu)M·h(^{-1})</td>
</tr>
<tr>
<td>( \beta_S )</td>
<td>Glutamate synthetase degradation rate</td>
<td>1</td>
<td>h(^{-1})</td>
</tr>
<tr>
<td>( \alpha_F )</td>
<td>FBI-GS production rate through binding of glutamine metabolites to GS</td>
<td>1</td>
<td>(\mu)M·h(^{-1})</td>
</tr>
<tr>
<td>( \gamma_Q )</td>
<td>Glutamine degradation rate by metabolism</td>
<td>1</td>
<td>h(^{-1})</td>
</tr>
<tr>
<td>( \beta_F )</td>
<td>FBI-GS degradation rate</td>
<td>1</td>
<td>h(^{-1})</td>
</tr>
<tr>
<td>( T_T )</td>
<td>Total TurA concentration</td>
<td>1</td>
<td>(\mu)M</td>
</tr>
<tr>
<td>( \alpha_T )</td>
<td>TurA activation rate by unbinding with FBI-GS</td>
<td>1</td>
<td>h(^{-1})</td>
</tr>
<tr>
<td>( \beta_T )</td>
<td>TurA inhibition rate by FBI-GS</td>
<td>10</td>
<td>(p)M(^{-1})·h(^{-1})</td>
</tr>
<tr>
<td>( \alpha_A )</td>
<td>maximum GOGAT synthesis rate</td>
<td>10</td>
<td>(\mu)M·h(^{-1})</td>
</tr>
<tr>
<td>( \beta_A )</td>
<td>GOGAT degradation rate</td>
<td>1</td>
<td>h(^{-1})</td>
</tr>
<tr>
<td>( \alpha_R )</td>
<td>maximum (P_{nasA}-yfp) synthesis rate</td>
<td>10</td>
<td>(\mu)M·h(^{-1})</td>
</tr>
<tr>
<td>( \beta_R )</td>
<td>YFP degradation rate</td>
<td>1</td>
<td>h(^{-1})</td>
</tr>
<tr>
<td>Parameters</td>
<td>Description</td>
<td>Value</td>
<td>Units</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>$\gamma_R$</td>
<td>YFP photobleaching rate</td>
<td>0.05</td>
<td>h⁻¹</td>
</tr>
<tr>
<td>$K_F$</td>
<td>Threshold of FBI-GS production</td>
<td>1</td>
<td>μM</td>
</tr>
<tr>
<td>$K_T$</td>
<td>Threshold of the inhibition of GOGAT by TnrA</td>
<td>1</td>
<td>μM</td>
</tr>
<tr>
<td>$K_R$</td>
<td>Threshold transcriptional activation of $P_{nasA}$ by TnrA</td>
<td>0.3</td>
<td>μM</td>
</tr>
<tr>
<td>$m$</td>
<td>Hill coefficient for FBI-GS production</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>$n$</td>
<td>Hill coefficient for the inhibition of GOGAT by TnrA</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>$k$</td>
<td>biofilm expansion rate</td>
<td>0.1</td>
<td>mm·h⁻¹</td>
</tr>
<tr>
<td>$r_0$</td>
<td>radius of area plated with initial $B. subtilis$ culture</td>
<td>1.5</td>
<td>mm</td>
</tr>
<tr>
<td>$\eta$</td>
<td>“freeze factor” of metabolic rate</td>
<td>2</td>
<td>mm⁻¹</td>
</tr>
<tr>
<td>$\epsilon$</td>
<td>metabolic scaling factor</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>$E_0$</td>
<td>Initial glutamate concentration</td>
<td>9</td>
<td>mM</td>
</tr>
<tr>
<td>$N_0$</td>
<td>Initial ammonium concentration</td>
<td>1</td>
<td>mM</td>
</tr>
<tr>
<td>$O_0$</td>
<td>Initial 2-oxoglutarate concentration</td>
<td>1</td>
<td>mM</td>
</tr>
<tr>
<td>$S_0$</td>
<td>Initial glutamine synthetase concentration</td>
<td>10</td>
<td>μM</td>
</tr>
<tr>
<td>$Q_0$</td>
<td>Initial glutamine concentration</td>
<td>0.6</td>
<td>mM</td>
</tr>
<tr>
<td>$F_0$</td>
<td>Initial FBI-GS concentration</td>
<td>0.7</td>
<td>μM</td>
</tr>
<tr>
<td>$T_0$</td>
<td>Initial free TnrA concentration</td>
<td>0.1</td>
<td>μM</td>
</tr>
<tr>
<td>$A_0$</td>
<td>Initial GOGAT concentration</td>
<td>2</td>
<td>μM</td>
</tr>
<tr>
<td>$R_0$</td>
<td>Initial YFP concentration</td>
<td>0.1</td>
<td>μM</td>
</tr>
</tbody>
</table>

**QUANTIFICATION AND STATISTICAL ANALYSIS**

FIJI/ImageJ, MATLAB, and Python were used for image analysis.

**Biofilm measurements**—For each biofilm movie, Radial Profile Extended plugin from FIJI (ImageJ) was used to determine intensity along the biofilm radius. A circle was manually fitted against the initial droplet of cells using the brightfield image of the first timepoint. This circle was used to find the coordinates of the center point of the biofilm. The plugin finds the mean intensities along the radial profile for each image in the time series. These mean intensities were normalized by the minimal and maximal intensities across all time points. For each time point plot, one line represents one biofilm. Maximum projection graphs were derived from data outputted from the radial profile plugin. The maximum intensity value across all points along the radius was found using custom Python code. This was repeated for all fluorescence channels and plotted using Matplotlib (Python). Average values and the 95% confidence interval were determined in Python.

The position of the wavefront was determined using $P_{hyperspank-cfp}$ “difference movies”. To obtain a difference movie, each frame was obtained by subtracting the pixel intensities of the previous timepoint from the current one. We then obtained and smoothed the radial difference profile and define the position of the wavefront using 50% of the maximum difference value. The wavefront speed was then measured using displacement of the wavefront position divided by time. The time between $nasA$ rings is determined by local
maxima of $P_{nasA\cdot yfp}$ time trace. The distance between $nasA$ rings is determined using the maximum projection image.

**Single cell tracking**—Custom MATLAB (MathWorks) software was written to track single cells over time. Phase contrast images were used to define an ROI for each cell for each image frame. The generated ROIs were then used to determine intensity in the fluorescence channels. A total of 67 cells from five independent experiments were tracked. Intensities were normalized within the same experimental set. The traces from different movie sets were then aligned at the time point where the normalized $P_{nasA\cdot yfp}$ fluorescence intensity was 0.2, which is considered above the background signal. Average values and the 95% confidence interval were calculated via Python.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgements**

We acknowledge Munehiro Asally, Tolga Çağatay, and Katherine Süel for helpful discussions. K.T.C. acknowledges support from the National Institutes of Health from grant T32GM127235. J.G.O. acknowledges support from the Spanish Ministry of Science, Innovation and Universities and FEDER (Project PGC2018-101251-B-I00 and CEX2018-000792-M), and from the Generalitat de Catalunya (ICREA Academia programme). G.M.S. acknowledges support for this research from the National Institute of General Medical Sciences from grants R01 GM121888 and R35 GM139645. G.M.S. is a Howard Hughes Medical Institute - Simons Foundation Faculty Scholar.

**References**


Highlights

- A bacterial segmentation clock patterns biofilm development
- Biofilms organize their nitrogen stress response into concentric rings
- The concentric ring pattern segments sporulation in space and time
- Bacteria use a clock and wavefront mechanism thought exclusive to vertebrates
Figure 1.
Nitrogen Stress in a *Bacillus subtilis* Biofilm is Organized in a Ring-Like Pattern
(A) Diagrams illustrate nutrient access (left) and expected metabolic stress (right) in a biofilm. Nutrient access is limited in the middle of a biofilm, and thus a biofilm is expected to experience higher metabolic stress in the middle.
(B) Illustration of the experimental set-up. A *B. subtilis* 3610 biofilm is grown on an MSgg-agar pad, and the biofilm development is monitored by time-lapse images using an inverted fluorescence microscope.
(C) Top view of a *B. subtilis* 3610 biofilm grown on a MSgg-agar pad for 3 days. Yellow dashed box indicates the microscope field of view. Scale bar, 1 mm.
(D) A double reporter strain is used to monitor nitrogen stress in a developing biofilm. *P_{nasA}*-*yfp* fluorescent signal is used to monitor nitrogen stress response together with *P_{hyperspank}*-*cfp* (*P_{hyp}*-*cfp*) fluorescent signal constitutively induced with 1 mM IPTG, which serves here as a control.
(E) Nitrogen stress response is monitored by *P_{nasA}*-*yfp* expression in a growing biofilm. YFP images (pseudocolored green) of 20, 28, and 36 hour time points are overlaid on the corresponding brightfield images. Scale bar, 1 mm.
(F) Constitutively induced *P_{hyp}*-*cfp* expression in the same biofilm shown in (E) serves here as a control for fluorescence measurements and cell density at each time point. CFP images (pseudocolored cyan) are overlaid on the corresponding brightfield images.
(G) Mean fluorescence radial profiles of P$_{nasA}$-yfp and P$_{hyp}$-cfp from three independent experiments (n=3). Each line represents a mean fluorescence profile over the radius of a biofilm. Profiles from (E) and (F) are shown with darker and thicker lines. See also Figure S1.
Figure 2.
A Clock and Wavefront Model for Nitrogen Stress in a Biofilm

(A) Circuit diagram illustrates the negative feedback loop in nitrogen stress response and how it regulates P_{nasA}·yfp stress reporter expression (R).

(B) Simulation results of the temporal dynamics of P_{nasA} expression (Stress reporter, R) from the negative feedback loop shown in (A) at a single cell level. Top is a simulation of a single cell showing the level of the stress reporter (R, a.u.) for each timepoint.

(C) The agent-based model considers cells positioned along one-dimensional radial axis (inset panel). In this model, only the outer-most cells (cells outlined in purple, clock cells) duplicate as the biofilm expands (each duplication is represented by two arrows). Nitrogen metabolism actively oscillates in these clock cells. These dynamics slow down in the “frozen” cells that are located inside the biofilm (gray area) due to limited nutrient availability. Thus, a gradient of nutrient availability forms a wavefront (black dashed line) that propagates with the biofilm expansion, leaving behind cells that are “frozen” in their metabolic state. The white-dashed line on the inset indicates the area of the initial drop of
culture applied to the agar pad in experiments, which, to be consistent, is discarded in the model.

(D) Simulations of $P_{nasA\cdot yfp}$ expression in the agent-based clock and wavefront model over time recapitulate experimental results (Figure 1E). The color bar shows normalized $P_{nasA\cdot yfp}$ expression (Stress reporter, $R$). Biofilm expansion is simulated starting from the white dashed line, mimicking the start of the biofilm growth from the applied initial culture of cells on agar pads.

See also Figure S2.
Figure 3.
Biofilms Can Produce Multiple Concentric Rings of Nitrogen Stress Response
(A) The clock and wavefront model suggests a scenario where a biofilm is intrinsically capable of generating multiple concentric rings by shortening the period of the clock.
(B) Diagram depicting external inputs, glycerol and glutamate, that can modulate the nitrogen stress response negative feedback loop in *B. subtilis*.
(C) Two-dimensional parameter space of glycerol and glutamate modulation. Blue shaded area indicates the parameter regime of oscillations. Different shades of blue represent different periods (hr). Black arrow illustrates the case of increasing both glutamate and glycerol concentration proportionally. See Methods for details.
(D) The period change over the proportional modulation of glutamate and glycerol along the line indicated in panel (C) with a black arrow.
(E) Hopf-bifurcation diagram showing the expression level of the stress reporter over the proportional modulation of glutamate and glycerol (Black arrow in panel (C)). Y axis shows normalized $P_{nasA}\cdot yfp$ expression (Stress reporter, R). The black part indicates the regime without oscillations. The red lines indicate the regime with oscillations and the maxima and minima of these oscillations.

(F) Agent-based model predictions of $P_{nasA}\cdot yfp$ expression (Stress reporter, R) over time, at fixed 10 times higher glutamate and glycerol input. Biofilm expansion is simulated starting from the white dashed line.

(G) Maximum projection of $P_{nasA}\cdot yfp$ expression (Stress reporter, R) at each position of a biofilm throughout the simulated time shows a multiple concentric ring pattern.

(H) Experimental results of $P_{nasA}\cdot yfp$ expression in a biofilm growing with three times the standard glutamate and glycerol concentrations. Fluorescence images (pseudocolored green) are overlaid on the corresponding brightfield images. Scale bar, 1 mm.

(I) Maximum projection $P_{nasA}\cdot yfp$ expression at each position in (H) throughout time is overlaid on the brightfield image at the last time point.

(J) Normalized intensity profile over the radius of the biofilm shown in (H) at each time point.

(K) Normalized intensity profile over the radius of maximum projections of seven biofilms from seven independent experiments. Green line represents the mean intensity profile, and green shade shows 95% confidence interval.

See also Figures S2–S4, and Movies S1 and S2.
Figure 4.
Experimental Validations of the Clock and Wavefront Model
(A) Circuit diagram showing the location of the two mutant strains tested: gltA deletion (ΔgltA) and tnrA point mutation (tnrA<sub>M96A</sub>). We note that the tnrA<sub>M96A</sub> mutant can no longer be inhibited by FBI-GS, yet can still bind downstream targets including P<sub>nasA</sub>. The gltA deletion mutant experiences less glutamine limitation (nitrogen stress), because the absence of gltA removes the inhibition of glutamine concentration.

(B) Maximum projection of simulation results of P<sub>nasA</sub>-yfp expression in a tnrA<sub>M96A</sub> mutant biofilm. This point mutation abolishes FBI-GS binding of TnrA without affecting its activity.
as a transcription factor (i.e. turns on \( P_{nasA-yfp} \) regardless of nitrogen stress status, see Methods). The area inside the white-dashed line representing the initial drop of cells is not simulated.

(C) Maximum projection of experimental data showing \( P_{nasA-yfp} \) expression in a \( tnrA^{M96A} \) mutant biofilm is overlaid on the brightfield image at the last time point. \( P_{nasA-yfp} \) signal is normalized to the \( P_{nasA-yfp} \) signal from the wildtype biofilm in the same experiment. Scale bar, 1 mm.

(D) Maximum projection of simulation results of \( P_{nasA-yfp} \) expression in a \( gltA \) deletion mutant. Absence of \( gltA \) removes glutamine inhibition and thus reduces \( nasA \) expression.

(E) Maximum projection of experimental data showing \( P_{nasA-yfp} \) expression in a \( gltA \) deletion mutant biofilm is overlaid on the brightfield image at the last time point. \( P_{nasA-yfp} \) signal is normalized to the \( P_{nasA-yfp} \) signal from the WT biofilm in the same experiment. Scale bar, 1 mm.

(F) Schematic showing the wavefront (white dashed line) that separates the metabolically active region ahead of the wavefront (violet) and the “frozen” region behind the wavefront (gray).

(G) The wavefront arises in simulations from a freeze term that scales all metabolic reaction rates according to the distance from biofilm edge. Violet color indicates reaction rates and saturates at 30% of maximum reaction rate. White dashed line indicates the “wavefront” defined by 50% of maximum reaction rate.

(H) Simulated \( P_{nasA-yfp} \) (Stress reporter, R) difference averaged from the region ahead of the wavefront (top, violet outlined) and behind the wavefront region (bottom, gray outlined). Color bar shows normalized \( P_{nasA-yfp} \) difference.

(I) The wavefront is experimentally visualized using \( P_{hyperspank-cfp} \) difference between two consecutive timepoints (pseudocolored violet). The white dashed line indicates the wavefront that discriminates ahead of and behind the wavefront. 50% of the maximum intensity value of the image is used to define the wavefront. The area outside the biofilm is shown in white and is determined using the brightfield image. Image taken at 31 hours Scale bar, 1 mm.

(J) Time traces of averaged \( P_{nasA-yfp} \) difference ahead of the wavefront (top, violet outlined) and behind the wavefront (bottom, gray outlined). \( P_{hyperspank-cfp} \) difference threshold was used to determine the region for analysis. Color bar shows normalized \( P_{nasA-yfp} \) difference. See also Figure S5.
Figure 5.
Oscillation of the Nitrogen Stress Response is Cell-Autonomous
(A) A clock and wavefront mechanism depends on the cell-autonomous oscillation of
the glutamine negative feedback loop, and thus a diffusional barrier would not affect the
ring pattern. However, if long-range extracellular signaling is required to generate the
nitrogen stress pattern, e.g. reaction-diffusion system, a diffusional barrier would alter
pattern formation.
(B) Experimental results of setting a diffusional barrier in a biofilm after the formation of
the $P_{nasA}$-yfp first ring. The barrier was inserted from the top to the bottom of the agar
at around 24 hours of the biofilm growth, after the first ring was developed and stayed
inserted until the end of the experiment. $P_{nasA}$-yfp images are normalized and overlaid on
the corresponding brightfield images. Scale bar, 1 mm.
(C) Illustration of the expected $P_{nasA}$ expression at the single-cell level based on the clock
and wavefront model. The model assumes cell-autonomous oscillations in nitrogen stress
response.
(D) Film strip of a representative single cell oscillating in its nitrogen stress response ($P_{nasA}$
expression) in a microcolony. $P_{nasA}$-yfp images (pseudocolored green) are overlaid on the
corresponding phase images. Scale bar, 2 μm.
(E) Normalized single-cell traces of $P_{nasA}$-yfp expression over time. Twenty traces from
three independent experiments are aligned by when each cell reaches a fluorescence
intensity threshold of 0.2. Green line represents the mean intensity profile and green shade
shows 95% confidence interval.
See also Figure S6.
Figure 6.
Sporulation Follows Nitrogen Stress

(A-C) Normalized single-cell traces of a double reporter strain with $P_{nasA}$-cfp (green) and $P_{spoIIR}$-yfp (magenta) in microcolonies. Traces from a representative cell are in darker and thicker lines and the corresponding composite cell images are above (scale bar, 2 μm). At the end of the first $P_{nasA}$ peak, a subset of cells undergoes sporulation (A). The cells that did not sporulate continue to oscillate (B-C). Traces from cells with one oscillatory cycle (A, n=15), cells with two cycles (B, n=15), and cells with three cycles (C, n=22) are from three independent experiments.

(D) Illustration of sporulation events at the single-cell level. The sporulation process is characterized by an increase in $P_{spoIIR}$ expression, which is an early marker of irreversible commitment to sporulation. Single-cell level analysis panels (A-C) show that the increase of $P_{spoIIR}$ expression succeeds the pulses of nitrogen stress.
(E) Film strip of $P_{nasA}$-*cfp* expression (pseudocolored green) at 24, 32, and 42 hr. Fluorescence images are independently normalized and overlaid on the corresponding brightfield images. Scale bar, 1 mm.

(F) Film strip of $P_{spoIIR}$-*yfp* expression (pseudocolored magenta) from the same double reporter biofilm shown in (E). Fluorescence images are individually normalized and overlaid on the corresponding brightfield images. At 24 hr, there is no expression above noise level.

(G) Maximum projection of $P_{nasA}$ expression in the biofilm shown in (E) from the beginning to 53 hr. The resulting fluorescence image is overlaid on the brightfield image at the 53 hr time point.

(H) Maximum projection of $P_{spoIIR}$ expression from the same biofilm as (F). Images are processed in the same way as (G).

(I) Normalized $P_{nasA}$ (left) and $P_{spoIIR}$ (right) intensity profiles over a radius from the maximum projection shown in (G) and (H).

(J) Illustration of sporulation events at the biofilm level. Sporulation becomes patterned following to the observed double ring nitrogen stress pattern.
### Key resources table

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemicals, peptides, and recombinant proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>Sigma-Aldrich</td>
<td>Cat#P3911, CAS: 7447-40-7</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Sigma-Aldrich</td>
<td>Cat#G5516, CAS: 56-81-5</td>
</tr>
<tr>
<td>L-glutamic acid monosodium salt hydrate (anhydrous)</td>
<td>Sigma-Aldrich</td>
<td>Cat#G5889, CAS: 142-47-2</td>
</tr>
<tr>
<td>Magnesium chloride hexahydrate</td>
<td>Fisher Scientific</td>
<td>Cat#BP214, CAS: 7786-30-3</td>
</tr>
<tr>
<td>Potassium phosphate monobasic</td>
<td>Fisher Scientific</td>
<td>Cat#BP362, CAS: 7778-77-0</td>
</tr>
<tr>
<td>Potassium phosphate dibasic</td>
<td>Fisher Scientific</td>
<td>Cat#BP363, CAS: 7758-11-4</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>Fisher Scientific</td>
<td>Cat#BP892, CAS: 67-03-8</td>
</tr>
<tr>
<td>Manganese chloride</td>
<td>Acros Organics</td>
<td>Cat#AC193451000, CAS: 13446-34-9</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>Fisher Scientific</td>
<td>Cat#BP510, CAS: 10035-04-8</td>
</tr>
<tr>
<td>Iron (III) chloride</td>
<td>Acros Organics</td>
<td>Cat#AC217090025, CAS: 10025-77-1</td>
</tr>
<tr>
<td>Zinc (II) chloride</td>
<td>Sigma-Aldrich</td>
<td>Cat#Z0152, CAS: 7646-85-7</td>
</tr>
<tr>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
<td>Sigma-Aldrich</td>
<td>Cat#IPTG-RO, CAS: 367-93-1</td>
</tr>
<tr>
<td>MOPS</td>
<td>Sigma-Aldrich</td>
<td>Cat#M3183, CAS: 1132-61-2</td>
</tr>
<tr>
<td>Rhodamine 123</td>
<td>Sigma-Aldrich</td>
<td>Cat#R3702-10MG, CAS: 62669-70-9</td>
</tr>
</tbody>
</table>

**Experimental models: Organisms/strains**

| B. subtilis NCIB 3610 amyE::Phyp-cfp, sacA::PnasA-yfp | This paper | N/A |
| B. subtilis NCIB 3610 amyE::PnasA-cfp, sacA::PspoIIR-yfp | This paper | N/A |
| B. subtilis NCIB 3610 sacA::PnasA-yfp, tnrA::tnrA M96A | This paper | N/A |
| B. subtilis NCIB 3610 sacA::PnasA-yfp, gltA::neo | This paper | N/A |
| B. subtilis NCIB 3610 sacA::PnasA(mut)-yfp | This paper | N/A |
| B. subtilis NCIB 3610 amyE::PnasA-cfp, sacA::PgltA-yfp | This paper | N/A |

**Oligonucleotides**

| GS1865: ctgaagcttGCTGTTCCTCCCCGCAAGC | This paper | N/A |
| GS1866: gctctagaCTGCGAGCCGGACTTTTTA | This paper | N/A |
| GS1867: cgggtaccCAGTTGAGACCGGACTTTTTACG | This paper | N/A |
| GS1868: egggaattCCGCGTCAATATCGACTGATG | This paper | N/A |
| GS1869: GCCCCCTCCAGAGCTTTTTCACCTGCGGATC | This paper | N/A |
| GS1870: GGAAAAAGCAGCTGGAGGAGGGGACGCTTAAAAAG | This paper | N/A |
| GS1871: GTGTAAGTTTTTGCGAAGGTATATGCCTTGAACATGC | This paper | N/A |
| GS1872: GCATTAAACGTTTCGCTTGTCTCCTTCCAG | This paper | N/A |

**Recombinant DNA**

<p>| ECE174-PnasA-yfp | Michael Elowitz lab, Caltech | N/A |
| ECE174-PnasA(mut)-yfp | This paper | N/A |
| ECE174-PgltA-yfp | This paper | N/A |</p>
<table>
<thead>
<tr>
<th><strong>REAGENT or RESOURCE</strong></th>
<th><strong>SOURCE</strong></th>
<th><strong>IDENTIFIER</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>pER449-trnA_M96A</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>ECE173</td>
<td>Bacillus Genetic Stock Center</td>
<td>BGSCID: ECE173</td>
</tr>
<tr>
<td>pDL30-PnasA-3xopt-cfp</td>
<td>Michael Elowitz lab, Caltech</td>
<td>N/A</td>
</tr>
<tr>
<td>ECE174-PspoIIR-yfp</td>
<td>Kuchina et al., 2011</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**Software and algorithms**

<table>
<thead>
<tr>
<th><strong>Original code</strong></th>
<th><strong>SOURCE</strong></th>
<th><strong>IDENTIFIER</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>This paper</td>
<td>MathWorks, 2020</td>
<td><a href="https://www.mathworks.com/products/matlab.html">https://www.mathworks.com/products/matlab.html</a></td>
</tr>
<tr>
<td>Python (Anaconda)</td>
<td>Anaconda, 2016</td>
<td><a href="https://anaconda.com">https://anaconda.com</a></td>
</tr>
<tr>
<td>FIJI</td>
<td>Schindelin et al., 2012</td>
<td><a href="https://fiji.sc/">https://fiji.sc/</a></td>
</tr>
</tbody>
</table>

**Other**

<table>
<thead>
<tr>
<th><strong>12-well plate</strong></th>
<th><strong>Corning Incorporated</strong></th>
<th><strong>Cat#353043</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass-bottom dish</td>
<td>Ted Pella</td>
<td>Cat#14027-20</td>
</tr>
<tr>
<td>Petri dish</td>
<td>Genesee Scientific</td>
<td>Cat#32-107G</td>
</tr>
</tbody>
</table>