Controlling the synchronization and symmetry breaking of coupled bacterial pili on active biofilm carpets

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eLife Assessment

This **important** study concerns the propagation of waves in bacterial biofilms, bridging active matter physics and bacterial biophysics. The experimental observations are **solid**, and the theoretical interpretation and model validation have been refined with revisions. This work will be of interest to microbiologists, biophysicists, and researchers studying collective behavior in biological systems.

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Abstract

In the low Reynolds number regime, active biological systems utilize nonreciprocal cyclic activities to achieve motility, as seen in the spinning of bacterial flagella and the beating of cilia. Coupling among these active mechanical components leads to synchronization, and emergence of metachronal waves. Here, we report that biofilms of *Pseudomonas nitroreducens* form active carpet-like surfaces textured with diverse topological defects, generating Mexican-wave-like collective behavior in which bacteria periodically lift up. On these active surfaces, nonreciprocally coupled extension and retraction activities of bacterial pili drives these collective oscillations. Surprisingly, this collective behavior exhibits left-right asymmetry across the biofilm driving unidirectionally propagating waves. We discover that this directionality is primarily governed by an aging-related frequency gradient across the biofilm. Leveraging these insights, we further demonstrate the ability to control the collective dynamics of these waves, including symmetry breaking, transitions from spiral waves into target and propagating plane waves by manipulating the elastic properties of biofilms. Overall, our findings illuminate the fundamental role of nonreciprocally interacting active components in regulating synchronization, collective dynamics, and symmetry-breaking phenomena in biological systems.

Introduction

At low Reynolds numbers regime, overcoming reversibility constrain to gain motility, is a challenging task which requires innovative solutions. Microorganisms utilize a variety of specialized active mechanical components to solve these difficulties by performing nonreciprocal motion[1–4]. Typical examples include the rotating helical flagella and beating elastic cilia. These active mechanical systems have the ability to execute cyclic motions, thereby breaking time-reversibility constraints[5]. In addition, due to the coupling among these active microstructures, complex synchronization phenomena also known as metachronal waves could emerge[6–8]. From these perspective coupling, synchronization and collective behaviors are intrinsically related and ubiquitous in nature. Such widespread dynamical processes are also biologically essential, as seen in cilia-driven mucus transport in lungs[9], nodal flow in developing embryos[10], central motor generators[11] and spinal cord development[12]. These intricate synchronization behaviors share the same underlying dynamical principles that surprisingly align with non-Hermitian physics of active systems[13–16]. All these active systems are capable of injecting and transferring energy across the system by breaking certain symmetries.

Recent advances, especially those in the field of active matter physics[17–22], have illuminated critical non-Hermitian features, such as chiral states where PT symmetry is broken. In this broken mode system could sustain the phase difference between fundamental fields or the modes which further trigger the directional energy transfer between the active compartments and generate travelling waves. Notably this non-Hermitian process is particularly driven by nonreciprocity in the coupled mechanical system[8,17,23]. Although non-Hermiticity has its roots in quantum mechanics[14,24] and the fundamental principles have been broadly studied in photonics[25], electronics, acoustics, optomechanics[26,27], superconducting qubits[28], trapped ions[29,30], single-spin systems[31], and in light-matter interactions[32]. This process is often achieved not only using strong nonlinearities, but also utilizing nonreciprocal interactions and coupling[33–35]. On the other hand, nonreciprocity is a very common feature in biological systems. Such as hydrodynamic interactions[36] or prey predator[37] relations could easily break action reaction symmetry and results in exotic collective behaviors. Extending the basic concepts from non-Hermitian physics to biological domain holds promise for shedding light on the fundamental principles of symmetry breaking in active and, more crucially, in complex biological systems.

Various biological models with specific active micromechanical components have been studied. The most notable biological platforms are ciliated epithelial cell[38], bacterial carpet[39,40], starfish embryo[41] social amoeba[42–44] and walking placozoa[45]. Addition to flagellum or cilia, bacteria could also use nano filaments known as type IV pili to gain motility on surfaces where the rotating flagella do not work effectively. Recent studies also highlighted the importance of pili controlling the collective behaviors of bacteria[46–49] and also large-scale oscillations in the form of biofilm.

During our recent experiments, we noted striking dynamic patterns and unidirectionally propagating waves on the surface of *Pseudomonas Nitroreducens (PN)* bacterial biofilms. These unexpected collective activities ranging from spiral to planar wave formations, interestingly aligned with recent predictions of active matter physics[23,50]. These activities are reminiscent of metachronal waves observed in various biological systems. We also observed that these collective activities are driven by the coupled pili activity of the bacteria. Recently, large-scale pili-driven activities and spiral waves were also identified in *Pseudomonas aeruginosa*[49]. The main distinction of our observed metachronal wave lies in its localized behavior on the surface and broken left-right symmetry across the biofilm, which we refer to as the active biofilm carpet. On these active surfaces bacteria periodically lifting up and forming Maxican-wave like dynamics. In this study, we aim to provide a comprehensive experimental approach including biological, physical and theoretical tools to be able to understand and control these collective behaviors and also the symmetry breaking process of these biological systems.

Bacterial biofilms develop into multicellular communities characterized by interdependent biological structures. Understanding the dynamics of these dense biological formations is vital, given that their collective actions can amplify their pathogenic potential. The collective movement of pili and its variability among strains offers a crucial foundation for understanding the influence of pili activities on pathogenicity. Moreover, pili is also a primary target protein for next-generation medications and antibacterial therapies. In essence, comprehending the effect of pili dynamics on the collective behavior of biofilms can greatly influence human health by preventing severe infections.

Results

Emergence of metachronal waves on active biofilm carpets

Bacterial biofilms are commonly used in various experiments particularly to investigate host-pathogen interactions. During our recent screen of biofilm library for nematode *C.elegans*[51], we observed that *Pseudomonas Nitroreducens* (PN, Materials and Methods) bacterial biofilms exhibit rhythmic activities approximately 10 hours post-inoculation (Figure1a-b). In their firing phase, faint propagating pairs of spiral wave patterns emerged on the biofilm's surface. These waves have a periodicity of around 2-7 minutes (Figure1c). We also determined that the visibility of these waves improves dramatically with oblique contrast and polarized light-based microscopy (Figure1a-b, Supplementary Video1-2). Later on, these waves were very dynamic and converged to various combinations of spiral, target and planar waves. The most striking feature of these waves is their broken left-right symmetries. They, often unidirectionally propagate from right to left (edge to the center) or radially in inward direction across the colony (Figure1a-b). Furthermore, the contrast of the wave's switches between the dark to bright sharp lines depending on the direction of propagation. This suggest that the front kink of these waves scatters the light asymmetrically (Supplementary Figure 1). We observed that these waves predominantly propagate on the biofilm's surface rather than its bulk region (Figure1e). This critical feature is particularly revealed by the nature of our optical imaging systems which is very sensitive to the surface topography. To validate this observation, we employed cytoplasmic GFP for imaging. Yet, no waves were detected in the green fluorescence channel (Supplementary Figure2). Similarly, when we labeled extracellular DNA with a dye, the waves remained unobserved in the fluorescence signal (Supplementary Figure2). Moreover, close-up imaging (100X) highlighted the bacterial displacement together with clear optical dark or bright contrast on the surface (Supplementary Video3). Imaging growing biofilm starting from a single bacterium clarifies that oscillations first starts from dense regions and gradually propagate through the entire colony (Supplementary Video4). Further motivated by recent studies[48,49], we also tested the *Pseudomonas aeruginosa* strain PA14 under the same conditions. Similarly, PA14 exhibited weak but comparable wave patterns, though within a shorter time window (Supplementary Figure4).

To delve deeper into these phenomena, our next focus was on the necessary media, given that the original experiments were conducted on *C. elegans* using Nematode Growth Media (NGM) plates (see Materials and Methods). Interestingly, we found that these activities were exclusive to NGM. Substituting NGM with a standard LB plate did not reproduce the same dynamics; instead, the bacteria grow rapidly. Yet, upon omitting yeast extract and replacing bactotryptone with bactopeptone, a major nutritional component, from the LB media PN resumed its activity (Supplementary Figure6). These findings underscore the significance of nutrient restriction as a primary factor. Remarkably, these observations are unexpected, but become evident due the use of NGM plate, which is not common in microbiology experiments.

Although PA14 generates similar waves we observed that PAO1, a close relative of PA14, failed to produce waves on both NGM and LB plates (Supplementary Figure5). Worth noting, PAO1 is also an important human pathogen causing *Cystic Fibrosis* (CF). Finally, through this comprehensive screening effort, we successfully identified a group of bacteria, offering the potential to examine various factors, from genetics to physical domain, influencing the emergence of these spiral waves. Both PAO1 and PA14 possess robust genetic toolkits, while PN strain display a clear and prolonged active carpet state for detailed physical investigations. The other critical difference between all these biofilms is the finger formation around the leading edge. Unlike PAO1, PN and PA14 form very strong instabilities leading unstable extensions (Figure1f).

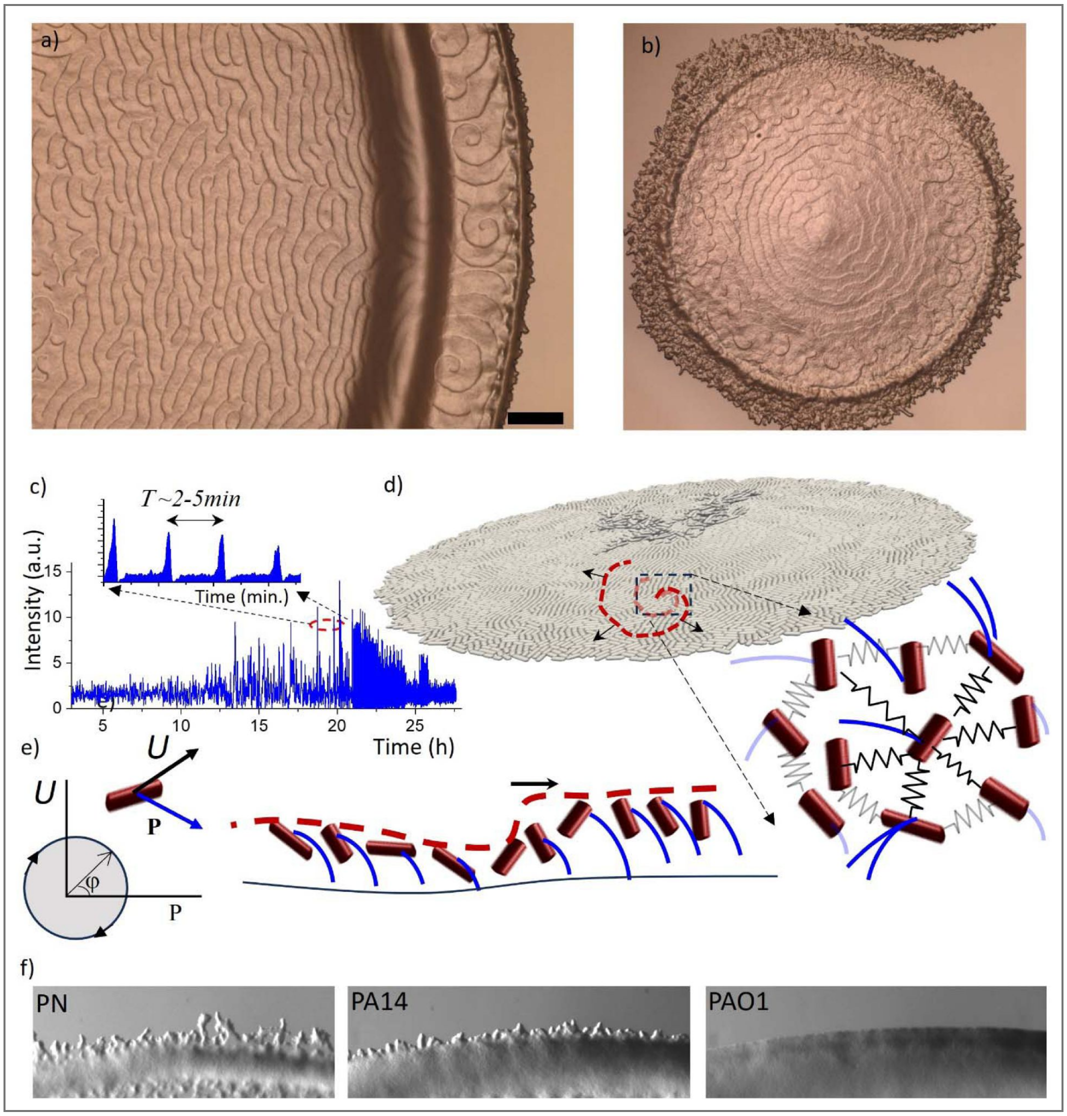


Figure 1. **Emergence of unidirectionally propagating waves on bacterial biofilms.**

(a-b) Optical imaging shows spiral, planar (a), and radially shrinking waves (b) propagating unidirectionally on biofilm surfaces. Left-right or radial symmetry is generally broken on growing biofilm surfaces. Scale bar 250 um. (c) Time response of optical scattering signal indicating the firing state of coupled pili dynamics. (d) Schematic representation of the coupling mechanism of pili on biofilm surfaces, modeled as an active solid. Red cylinders represent the bacteria in the biofilm. The oscillatory extension and retraction of pili act as active units. (e) Schematic representation of the collective behavior of elastically coupled active biofilm surfaces, characterized by local displacement (//) and pili polarization (P) undergoing limit-cycle oscillations. Propagating waves remain localized on the surface and travel toward the direction of the sharp rising edge. f) Optical images of the leading edge of the growing biofilm with fingering instabilities. PN and PA14 show strong fingers compared to PAO1.

To gain deeper insight into the mechanisms underlying wave formation, we imaged the dynamics of individual bacteria from the fingering regions toward the center of the biofilm. This distinction is critical because, unlike the biofilm center, the edges do not generate waves. We observed that bacteria near the fingering regions remain motile and exhibit collective flow. In contrast, bacteria at the biofilm center are surface-attached and undergo periodic lifting motions. This behavior strongly resembles Mexican-wave dynamics (Supplementary Video5, 6).

We further found that the central region of the biofilm is mechanically more elastic (Supplementary Figure3), whereas the edge regions—where wave formation is absent—are motile. These observations suggest that gradual biofilm maturation is a key factor that transforms motile bacteria into a periodically moving but spatially constrained state. Consistent with this picture, the PAO1 strain, which has a strong biofilm-forming capability, completely suppresses surface oscillations. In contrast, the PA14 strain exhibits intermediate behavior, sustaining a partial transition between motile and locally constrained dynamics. Remarkably, signatures of this transition and wave generation are already detectable at the earliest stages of finger formation.

We then shifted our attention to the genetic distinctions between PN, PA14 and PAO1. Initially, we first explored the contribution of bacterial pili and flagella as an active mechanical structure. We found that PA14:ΔPilB and PA14:ΔPilA mutations eliminated the waves but not PA14:ΔFilK (Supplementary Figure4). This suggests that activity is exclusively originating from pili dynamics and the original dynamics is similar to the recent work[49], but activities are localized to the surface to the transition from motile to biofilm forming state. We then explored the potential of hyper piliation, which might amplify the coupling of pili on the biofilm surfaces. Yet, the PAO1 ΔPilH mutant, known for its polarized and hyper piliation condition[46,47], did not manifest the waves but provided only small fluctuations on the surface (Supplementary Figure5). Then, we pinpointed that a significant variation was in the pili subunit groups[52]. While PA14 possesses group 3 Pilin, PAO1 has group 2 Pilin. It's essential to highlight that all these bacteria have type IV pili, though they might be categorized into separate groups. Currently, our hypothesis posits that diverse pili types might exhibit varied physical characteristics such as elasticity or rigidity. Our understanding of PN at the genetic level remains limited but it shares strong similarity with PA. Nonetheless, the various genome sequences of PN are accessible. Our analysis of their pili related genomic region revealed a resemblance to the group 4 and 5 pili (Supplementary Figure7). All these firing pili groups 3 and 4, 5 have additional accessory genes in the genome and they may contribute to the expression or folding of these pilins. Together with the unstable edge fingering (Figure1f), the comparison of PN and PA strains support the idea that group of pili and particularly pili expression play a critical role in driving waves on these bacterial biofilms.

Modelling of propagating spiral waves

To get more intuition about the dynamics of the waves and emergence of the broken symmetry across the biofilm, we next focused on the mathematical modelling. From an active matter perspective, spiral waves can emerge in non-equilibrium and excitable media[53–55] as well as in coupled oscillatory systems with delays[56]. Despite these systems exhibiting similar wave patterns, the internal mechanisms that trigger the waves vary significantly. Recent studies particularly on the concept of nonreciprocity provides comprehensive approach[23,50]. It is important to note that coupling between active mechanical units could introduce nonreciprocity into the system due to local symmetry-breaking processes. Inspired from the previous studies on the dynamics of cilia[17,57], and recent robotic system[58] we proposed that coupled active pili system could also show similar limit cycles oscillations defined by bacterial displacement ($\mathbf{U}$) and the polarization of the bacterial pili ($\mathbf{P}$) (Figure1e). We note that combining phenomenological field models with more detailed elastic solid models could provide a more comprehensive framework for understanding how the elastic properties of the biofilm drive these collective oscillations. To study these phenomena, we first utilized a minimal phenomenological phase field model that includes a nonreciprocal coupling term. The oscillatory dynamics of the model are driven by oscillatory extension and retraction processes. The Kuramoto based model[23,56,59–61], which effectively captures the phase dynamics of coupled oscillators, is extended here by incorporating a delay term (α) that disrupts the odd symmetry—representing action and reaction symmetry between interacting oscillators—and enriches the system's dynamic repertoire. This modified model is described by the equation:

$$\frac{d\emptyset_i}{dt} = w_0 - b\sin\left(\emptyset_i\right) - K\sum\nolimits_j \sin\left(\emptyset_i - \emptyset_j - \alpha\right)$$

Here, (ø represents the local phase of individual pili oscillations (The phase of confined parameter space defined by **U** and **P**, Figure 1e). We assume interactions only among nearest neighbors, where W_Q represents the uniform intrinsic oscillation frequency of an isolated oscillator. The term α specifically break odd interaction symmetry between pili, facilitating the formation of travelling waves. Note that traveling waves indicate this broken PT symmetry between these fields. It's important to emphasize that the detailed biophysical mechanisms behind this nonreciprocity remain unclear. Several possibilities, such as force relaxation dynamics or hydrodynamics interactions, could also result in similar nonreciprocal behavior[62]. Our phenomenological model accurately captures the principal features of these phenomena. While reciprocally coupled oscillators (α = 0) tend to achieve a globally synchronized state (Figure 2a), nonreciprocity favors the formation of traveling waves (Figure 2b-c, Supplementary Video7, Supplementary Figure8). The excitability term (**b**) shapes the pulsative nature of the waves (illustrated in Figure 2d-e) by amplifying the effects of specific phases within the coupling mechanism[56,63,64].

$$\partial_t \boldsymbol{U} = \pi\boldsymbol{P} + \boldsymbol{F}_e$$

$$\partial_t \boldsymbol{P} = \left(\boldsymbol{P} \times \partial_t \boldsymbol{U}\right) \times \boldsymbol{P} + \frac{1-\boldsymbol{P}^2}{2}\partial_t \boldsymbol{U} - D_r \boldsymbol{P}$$

Next, we turned our focus to continuum simulations to capture the essential properties of these dynamic processes (Supplementary Video8). In our previous experiments, we observed that changes in the elastic properties of the colony during the transition from a motile state to a biofilm-forming state are critical. The recently developed active solid model provides a broad and robust framework for studying collective behaviors of interacting elasto-active components, spanning from robotic systems to human crowds[58,65]. The core idea of this model is based on two nonreciprocally coupled fields: displacement (**U**), representing bacterial displacement within the biofilm lattice, and polarization (**P**), representing the orientation of pili, which exert active forces whose direction can be modulated by the displacement field (Figure1d). The dynamics of the active biofilm lattice can be described by coupled equations, where $\mathbf{F_e}$ denotes elastic force and D_r represents polarization relaxation. The underlying rationale of these equations is that retracted pili exert active forces on bacteria, which are elastically coupled to the biofilm structure. Local biofilm displacement can guide the growth direction of pili extension, influenced by local deformation. Importantly, since pili are anchored to the substrate, their orientation dynamically adjusts based on extension processes. Our simulations demonstrate that the active solid model successfully captures critical dynamical phenomena, such as the formation of spiral and planar waves (Figure 2 f-g). As anticipated, fields **U** and **P** develop a characteristic phase difference, indicative of limit cycle oscillations. Moreover, analysis of vector configurations around defect cores reveals directional reversals of displacement **U** on opposite sides of defects, highlighting the fundamental mechanism underlying defect formation, where **U** diminishes, and the phase (ø) becomes undefined (Supplementary Figure9). We should note that traveling waves indicate broken PT symmetry between these fields triggered by nonreciprocity, with spiral waves serving as a classic signature of this phenomenon. A further transition from spiral to planar waves reflects an overall asymmetry in the frequency profile, which is not directly related to PT-symmetry breaking.

Controlling the transition between the waveforms on active biofilm carpet

Extensive parameter testing has revealed that our models not only capture the emergence of spiral waves but also predicts transitions into target and plane wave solutions which we commonly observe in grooving biofilms. This transition is particularly emerge around the edge of the plate

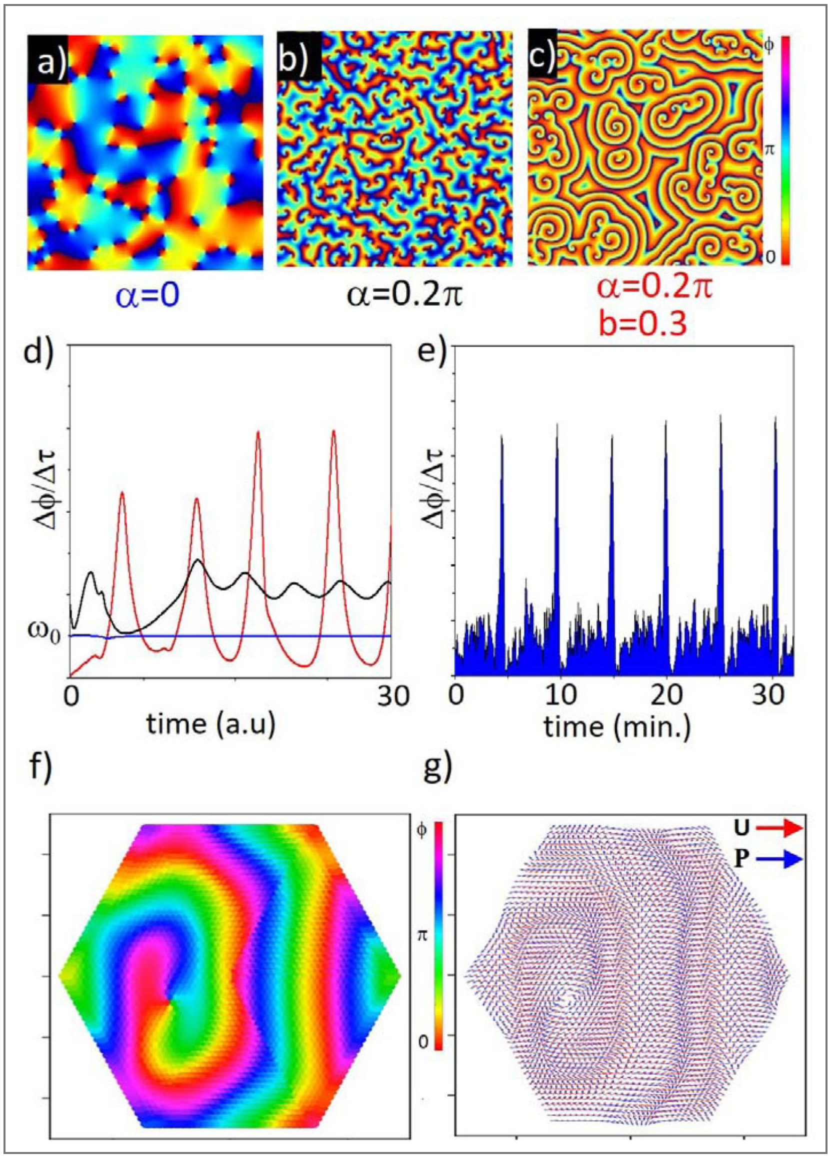


Figure 2. **Numerical modeling of coupled pili dynamics as an active carpet.**

(a-c) Numerical simulation results based on the nonreciprocal Kuramoto phase-field model. Nonreciprocal coupling term ($_\alpha$) among bacteria drives the emergence of spiral waves, while the excitability term (b) of the mechanical system induces pulsatile behavior. (d) Numerical simulations of local oscillation frequencies under varying conditions ($_\alpha$ = 0, $_\alpha$ = 0.2$_\pi$, and b = 0.1$_\pi$). (e) Experimental measurements of pulses on biofilm surfaces confirming pulsatile responses. (f-g) Numerical simulations of elastically coupled biofilm structures using the active solid model. Active solids exhibit large-scale spiral and planar wave formations. Displacement (U) and pili polarization (P) fields highlight the essential phase difference necessary for wave propagation and limit-cycle oscillations.

where the drying process is gradually modifying the agar plate. During the transition to target waves, two oppositely spinning spiral waves with topological charges of +1 and -1 merge, forming a symmetrically expanding target wave. Subsequently, fast oscillating plane waves progressively dominate the entire simulation domain (Figure3 a-c, Supplementary Video7).

Motivated by our simulation results and experimental observations, we hypothesize that our active carpet system could also facilitate similar transitions between different forms of traveling waves[66,67], offering a means to externally control the dynamics of the system and may explain the details of broken symmetries. We observed that adding a water droplet to the biofilm surface led to the transformation of spiral waves into target and planar waves (Figure 3d-f). To recover the spiral waves, we increased the surface temperature by gently heating the system. As expected, with the temperature rise, the leading edge of the planar waves first became noisy, and multiple target waves randomly appeared then finally converge to spiral waves (Supplementary Video9). These findings are in interesting similarity with recent theoretical predictions in different nonreciprocal systems[50,66].

Additionally, by incorporating the water-soluble polymer polyethylene glycol (PEG) into the biofilm, we managed to not only alter wave propagation patterns but also direct the movement of inward growing target waves. Intriguingly, the addition of small drop of PEG modified the intrinsic oscillation period of the pili dynamics, creating a radial gradient profile largely due to the slow evaporation rate and uneven deposition of PEG on the surface. This inward wave guidance is particularly controlled by frequency gradient (Figure 4a-d). The center has slow oscillations than the edge. This gradient profile is the critical feature to understand the basics of broken symmetry observed in the circular biofilms. In naturally growing colony oscillation frequency is also varying (Figure 4a-b). We hypnotize that this variation is age dependent where the center of the colony is slowing down due to change in elastic properties of the biofilm. Before delving deeper into this interesting symmetry-breaking process, we investigated how the aging of the biofilm affected the oscillation dynamics. This is because symmetry breaking occurs at late stage where the aging is critical. To do so, we first measured the oscillation periods as the uniform colony aged. We observed that the periods of these spiral waves continuously increased (Figure 5a-b). These results suggest that the intrinsic oscillation dynamics of the Pili decreased, likely due to biofilm formation which changes the elastic properties of the colony, during first 24 hours.

Further, we examined the growing biofilm starting from a thin inoculation strip, we reproducibly achieved a broken left-right symmetry for plane waves across the colony (Supplementary Video10). The waves propagated from the edges toward the center. This asymmetry became particularly pronounced a few days after inoculation, during which the colony edges continued to grow while the center remained stationary (Figure 6a, b). To finally confirm this observation, we numerically solve the coupled oscillators under varying frequency. Similarly, we found similar left-right asymmetry where the planar waves propagating towards the slower region of the biofilm center (Figure 6c, d, Supplementary Video11).

Defect dynamics controlling the transition between spiral to target waves

To better understand the dynamics of the transition between different form of the waves we focused on numerical simulations. We noticed that the motility of defects is the crucial parameter governing the transition between spiral, target, and planar waves varying the moisture content provides an effective and experimentally accessible control this motility. Our analyses revealed that spiral defect cores can move and merge to form target waves or annihilate entirely—processes that we also observe experimentally. This rich dynamical behavior underscores the importance of elasticity in shaping pattern transitions. First, we compare defect dynamics in both Kuramoto-based simulations and the active solid model. Both systems exhibit similar defect-survival behavior. As shown in Supplementary Figure10, pairs of unlike (+/−) defects can stably persist only at high nonreciprocity. We further quantify this behavior by plotting the separation distances between unlike defect pairs and find that short-range defect separations are possible exclusively in the high-nonreciprocity regime (Supplementary Figure11). This high-

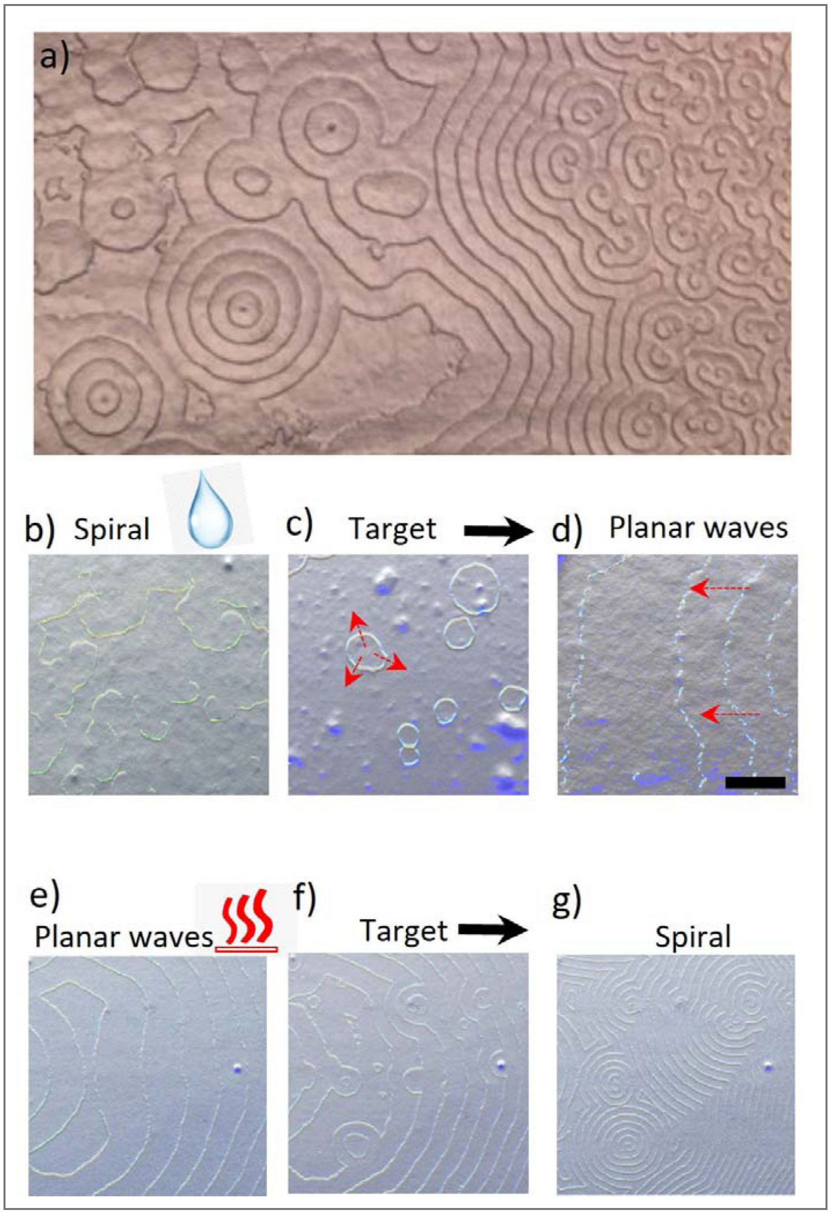


Figure 3. **Controlling transitions between spiral, target, and planar waves.**

(a) Experimentally observed transitions from spiral waves to target and planar waves spontaneously emerge across the plate. Pairs of spiral waves merge, forming topologically neutral target waves, which eventually give rise to planar waves dominating the biofilm surface. (b-c) Optical imaging of biofilm surfaces demonstrating similar controlled transitions between spiral, target, and planar waves experimentally triggered by adding a water droplet. Red arrows indicate wave propagation direction. (e-g) Controlled recovery of spiral waves achieved by heating biofilm surfaces, removing excess moisture, and facilitating re-emergence of spiral waves around a specific inhomogeneities.

Figure 4. **Controlling dynamics of inward propagating waves.**

(a) Optical imaging of inward propagating waves within a circular biofilm structure. (b) Period of pili oscillations decreases toward the colony center. (c) Application of a small droplet containing PEG creates a radially varying period profile, guiding inward wave propagation, capturing similar wave the dynamics on naturally growing radially symmetric biofilms. Error bar shows the S.D, N= 5 measurements.

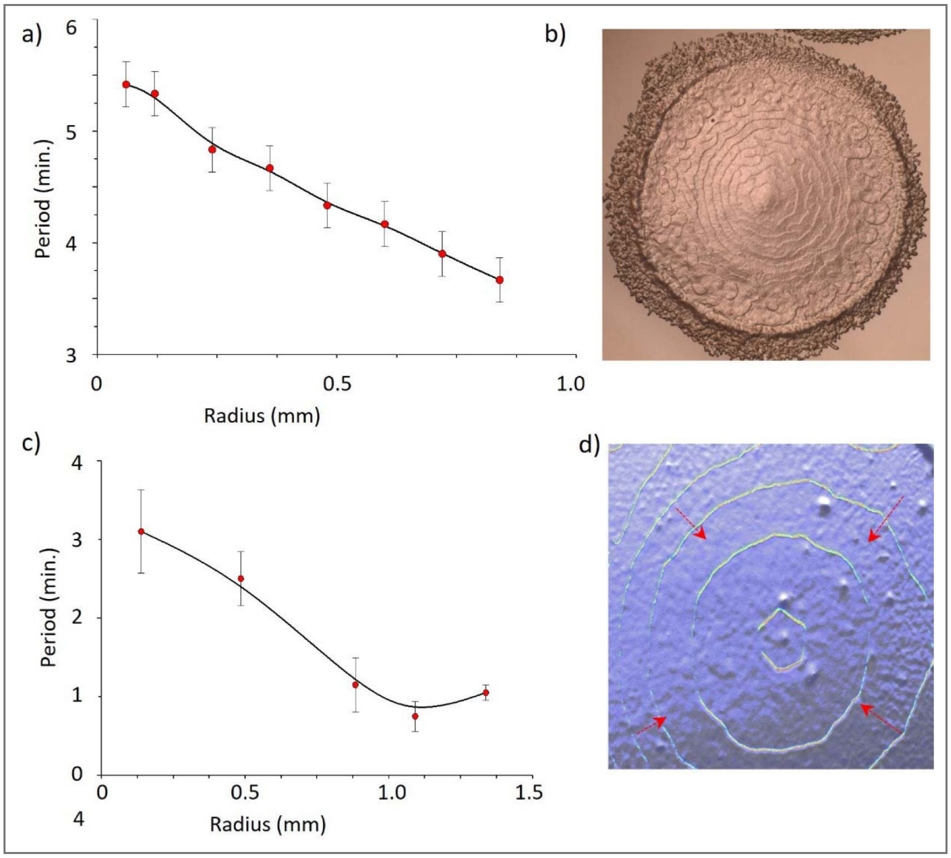
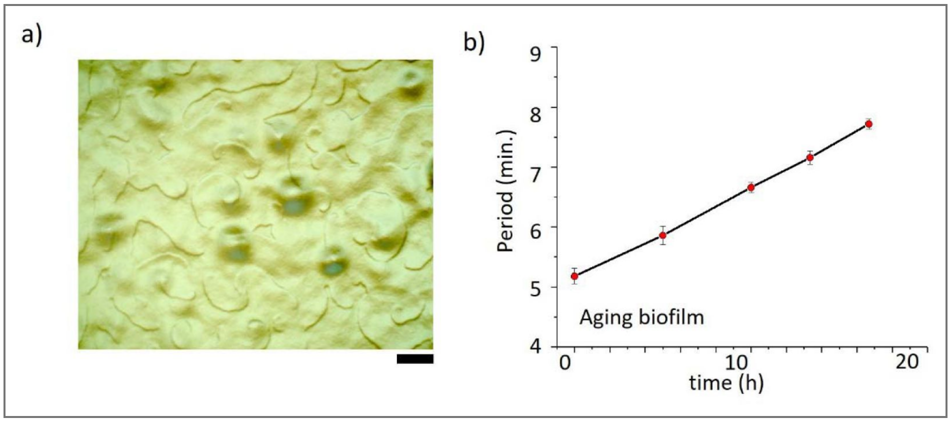


Figure 5. **Age-Dependent Dynamics of Oscillations.**

a) Sample image showing multiple pairs of spiral waves on a uniform biofilm surface. Scale bar: 100 μm. b) Period of the oscillations increases as the biofilm ages. Error bar shows the S.D. N= 10 measurements.



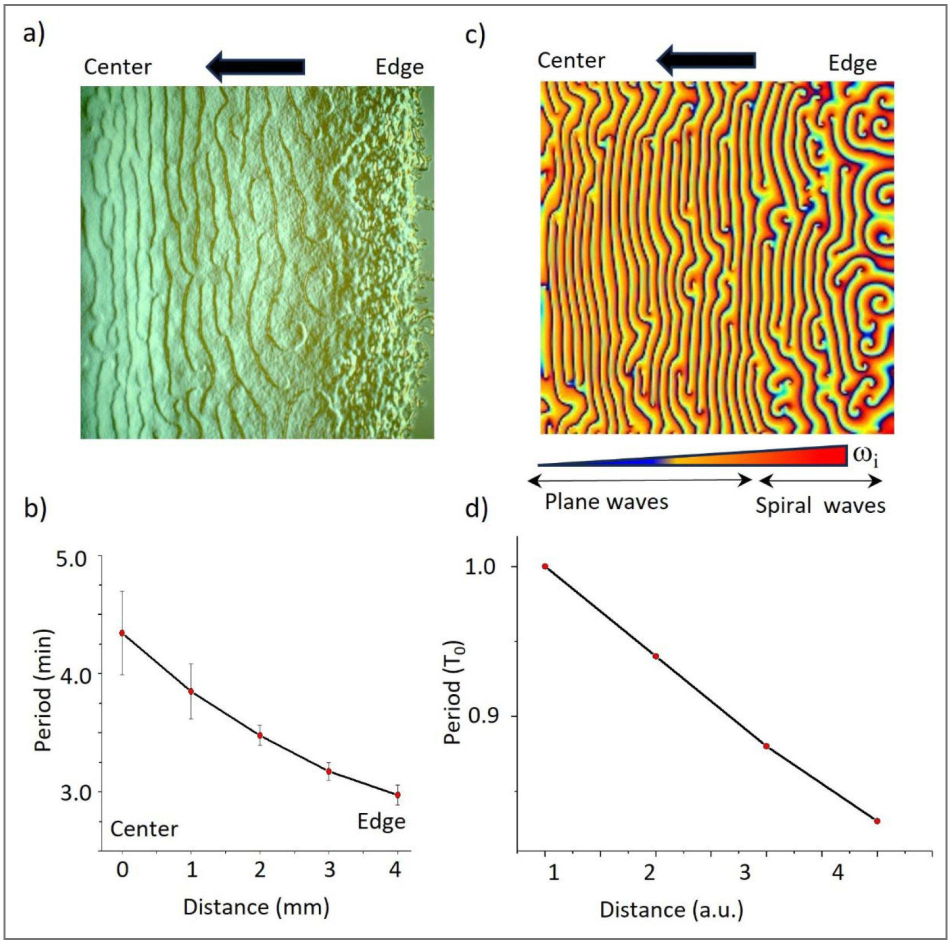


Figure 6. **Left-right asymmetry in naturally growing biofilms.**

(a) Representative image of a bacterial biofilm at a late growth stage (3 days post-inoculation). Metachronal waves propagate towards the biofilm center, with the leading edge displaying chaotic dynamics. Following growth cessation, spiral waves gradually converge into planar waves propagating inward. (b) Oscillation period increases towards the biofilm center. Error bar indicates the S.D. N= 10 measurements (c-d) Numerical simulation demonstrating the formation of planar waves and the left-right symmetry-breaking process, driven by a spatially varying intrinsic oscillation frequency(d), capturing dynamics observed in naturally growing biofilms.

nonreciprocity regime corresponds to the dry biofilm state. Increasing moisture reduces elasticity, leading to the loss of stable defect dynamics and promoting the annihilation of unlike defect pairs, which in turn drives the system toward target-wave formation and ultimately planar waves. Conversely, heating the biofilm removes water, enhances elasticity, and increases the system's ability to sustain closely separated defect pairs. Experimentally, we further observe that removing water by heating enhances surface nonuniformities, which readily trigger defect-pair formation (Supplementary Video9). To investigate this mechanism, we performed additional simulations in which local nonuniformities were introduced (Supplementary Video12-13). Consistent with experiments, defect-pair generation occurs only at high nonreciprocity, where pairs of unlike defects can be stably maintained. Experimental observation (Supplementary Video9) also show that surface nonuniformities on the biofilm surface similarly trigger the formation of closely separated defect pairs.

Controlling left-right asymmetry

As a final step we focused on how to control the symmetry breaking and dynamics of metachronal waves. We noticed that temperature adjustments also reversibly effect the oscillatory behavior of the waves which increased the oscillation frequency (Figure 7a, b). This suggest that temperature could be also used as a complementary technique to dynamically guide the wave propagation by controlling the frequency gradients. To do so we embedded metallic pipes in the bulk agar plate and tuned the local temperatures (Supplementary Figure13). Setting different temperatures in these pipes creates temperature gradient ($\nabla T \sim 10°C/cm$). This gradient profile converted spiral waves quickly to planar waves, propagating from warmer (31°C) to cooler (21°C) areas. Similarly, when we switched the temperature profiles wave propagation also reversed (Figure 7c-d). This result indicates that spatial temperature variations can effectively dictate wave propagation directions. These comprehensive observations confirm that the developed frequency gradients of intrinsic Pili oscillations is the critical feature breaking symmetry and controlling the propagation directions of the waves.

Discussion

Synchronization is a ubiquitous phenomenon in biological systems. A classic hallmark signature of this dynamical process is global synchronization, where all components of the system tend to merge in phase, as observed in fireflies, clocks and metronomes. Hydrodynamics or intrinsic activities of the interactions, however, introduce nonreciprocity into these tightly coupled systems, fundamentally altering the synchronization process. This action reaction asymmetry drives the emergence of large-scale propagating waves, often emerge as spiral waves in two dimensions. Remarkably, diverse systems from proteins in cell membrane to social amoebae exhibit similar dynamics, highlighting the special importance of these waves in biological systems particularly due to their transport capabilities.

From a symmetry perspective, nonreciprocity is the key feature in understanding these phenomena. Recent advancements in active matter physics have bridged various seemingly unrelated fields around this concept. Particularly, non-Hermitian physics, including PT-symmetry, provides a beneficial macroscopic metric for understanding a system's response through the classical formalism borrowed from quantum physics and optics. Despite the diverse nature of the dynamic processes and the varying types of interactions—from predator-prey dynamics, specific chemical reactions, to elasticity of active metamaterials—the common feature across these systems is to maintain phase difference between the fundamental fields that support limit cycle oscillations enabling collective motility and transport capabilities to the interacting active system.

We have observed that bacterial biofilm surfaces also known as active carpet exhibit similar dynamics due to coupled and cyclic pili extension and retraction activities. Although these nanoscale activities make the biophysical mechanisms complex and demanding to elucidate, the fundamental characteristic of the process remains consistent: the cyclic motion of bacterial pili forms limit cycle oscillations in phase space to disrupt local or action-reaction symmetry among the interacting components.

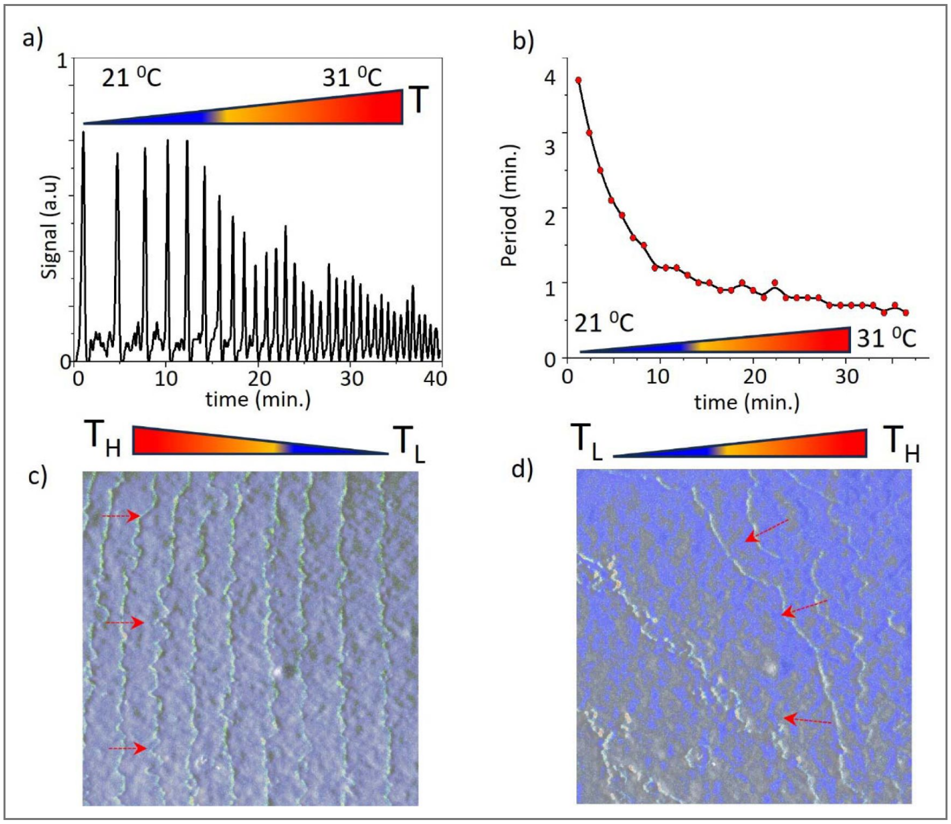


Figure 7. **Temperature-Controlled Dynamics of Metachronal Waves generating left-right asymmetry.**

a) Optical pulses from spiral waves on the biofilm surface as temperature increases from 21°C (T_L) to 31°C (T_H). b) An increase in temperature raises the oscillation frequency c, d) Controlling the propagation of the waves by creating a temperature gradient. Waves propagate from the warmer area (fast oscillating, T_H) to the colder region (slow oscillating, T_L). d) Reversing the temperature gradient changes the direction of propagations of the waves.

Using a phenomenological model, we have concentrated on identifying critical parameters that control the system dynamics and synchronization. We have also developed methods to manipulate and control the collective dynamics and propagation of metachronal waves by adjusting these critical parameters. From the perspective of active matter, the controllability of densely coupled active mechanical components is crucial, especially at low Reynolds numbers where controlling turbulence and managing long-range transport is vital. Biological systems can effectively address these challenges and precisely control active and programable transport[68]. A deeper understanding of the underlying principles and critical symmetries governing this controllability could provide new insights into the complexities of biological systems. We speculate that these waves could promote active diffusion of oxygen and DNA. Further biophysical investigations are needed to delve deeper into these fascinating collective behaviors and its biological significance. Furthermore, the dynamical similarities between non-Hermitian physics and PT symmetry and the metachronal waves due to nonreciprocity are remarkable. Finally, we also hypothesize that the concepts developed in no-Hermitian physics could bring new perspective to dissect the complexities of these collective behaviors. Finally, our findings also raise several questions, particularly regarding the biological significance of these waves in the physiology of the biofilm. Rhythmic activities[69,70] play a critical role in bacterial communities. We hypothesize that this synchronization may facilitate rhythmic behavior across the biofilm or enable expression and the transport of specific biomolecules under stressful conditions. Further studies are needed to elucidate the mechanisms underlying this emerging phenomenon.

Materials and Methods

Analysis of Pilin Proteins and Accessory Genes

Genetic analyses were conducted using the complete genome sequence of Pseudomonas nitroreducens strain L4 chromosome[71] (NCBI Reference Sequence: NZ_CP120376.1). The reference genomic region between pilB and tRNA-thr was specifically compared.

Imaging System

Time-lapse imaging was performed using multiple microscopy techniques, selected for their capability to visualize surface topography effectively. We observed that oblique and polarized illumination techniques significantly enhanced the visibility of surface waves. During the evaporation process, the roughness of the biofilm significantly varies. During the evaporation process, the roughness of the biofilm varies significantly. We observed that oblique illumination is more effective on rough surfaces, whereas polarized imaging provides clearer results on flat surfaces. Oblique imaging was primarily conducted using Zeiss Smartzoom digital microscopes and Nikon Stereo SMZ18 microscopes equipped with contrast illumination. Additional polarized imaging utilized a Zeiss Axio Imager M2m. Successive images were captured at intervals of 10 seconds. Fluorescence imaging for GFP was conducted using 488 nm blue-light excitation with GFP emission filters, and DNA staining was visualized using RFP emission filters and imaged with an Andor EMCCD camera. The typical optical magnification was 10X.

Image Processing

To enhance wave visibility, successive images taken at 10-second intervals were subtracted, and the resulting false-colored difference images were superimposed onto the original ones. The time-dependent responses of surface pulses were analyzed using ImageJ software.

Bacterial growth and Preparation

The Pseudomonas nitroreducens (PN) bacterial strain used in this study was initially isolated from environmental samples and identified through 16S rRNA sequencing. Verification was performed through comparison with the corresponding strain obtained from DSMZ. For optical imaging, PN bacteria were grown overnight in LB broth at 21 °C on a shaker, and subsequently, 100 µl drops of bacterial suspension were placed onto 10-cm, 1-day-old, 1.4% solid agar, NGM plates. To ensure slow evaporation of excess moisture, humidity was maintained at approximately 80%, and plate lids were partially left open. Nematode growth medium (NGM) plates were prepared following the standard protocols.

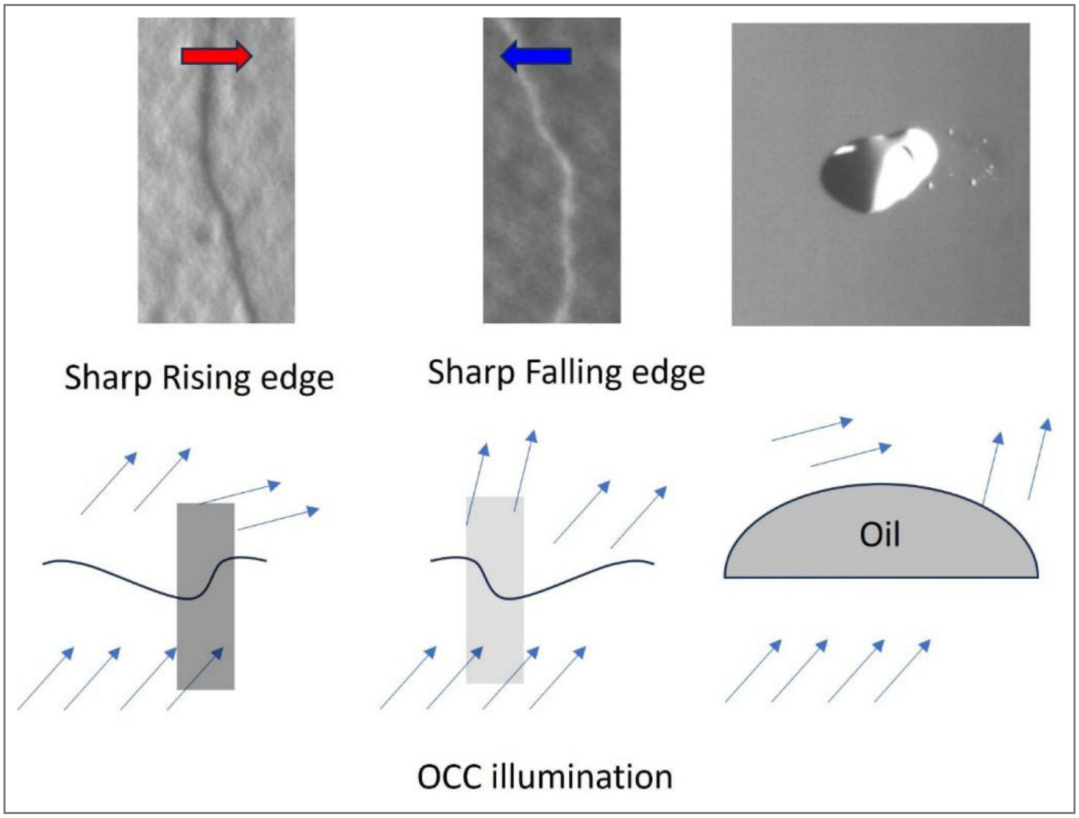
Numerical Simulation

Numerical simulations based on the Kuramoto model were performed using custom-developed MATLAB code with finite difference algorithms (*https://github.com/akocabaslab/kuramoto-oscillators*). The original code for the active solid model was obtained from prior literature and modified for improved visualization. Through extensive parameter exploration, we determined that large lattice structures with fixed boundary conditions effectively reproduced spiral and planar wave dynamics.

Strain	Parent	Operation	Genotype
BAK132	PN	Isolated from environmental samples, transformed pUCP18-MCSgfpmut3	*ampR*
BAK133	PA14	Received from F. M . Ausubel lab	
BAK134	LD2222	Received from Lars Dietrich Lab[72]	PA14 ΔpilB
BAK135	LD2221	Received from Lars Dietrich Lab[72]	PA14 ΔflgK
BAK136	PAO1 PilH	Received from Joanne Engel	ΔPilH
BAK	PAO1	Received from DSMZ 10145GFP	

Table 1. **List of strains used in this study.**

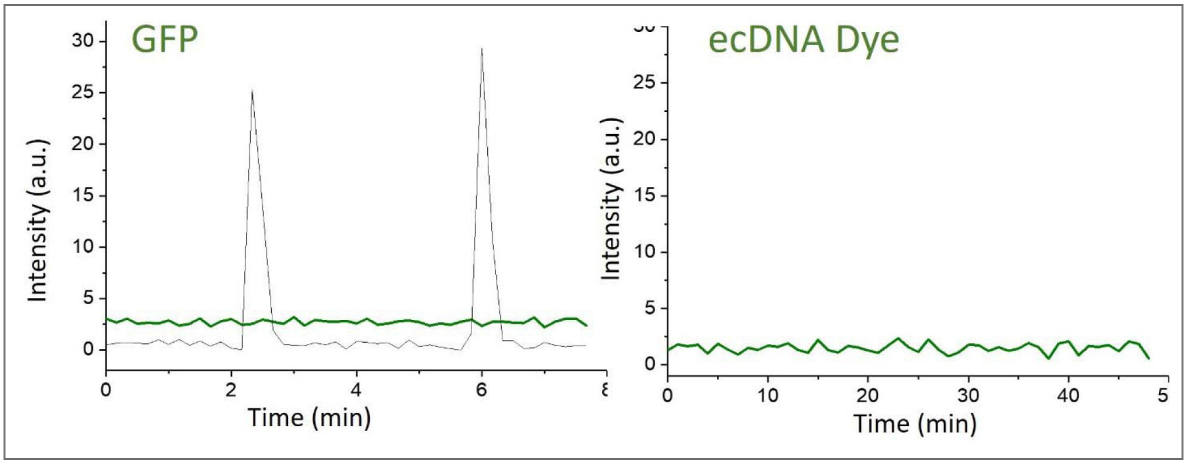
Supplementary figures



Supplementary Figure 1. **Asymmetric surface deformation drives directional propagation.** OCC imaging provides clear dark and bright optical contrasts, originating from oblique illumination and asymmetric scattering by surface waves. An oil droplet was used to investigate the shape-dependent propagation mechanism. Under oblique illumination, sharp rising edges generate dark contrasts, while falling edges create bright contrasts. Sharp edges correspond to rapid pili retraction, followed by gradual recovery.

Supplementary Figure 2. **GFP labeling and extracellular DNA staining were used to verify the origin of the optical scattering, specifically to distinguish between surface versus bulk oscillations in the biofilm.**

Neither fluorescence imaging method revealed oscillatory signals. Simultaneously recorded gray signals, indicating the presence of original waves on the biofilm, were superimposed for comparison.

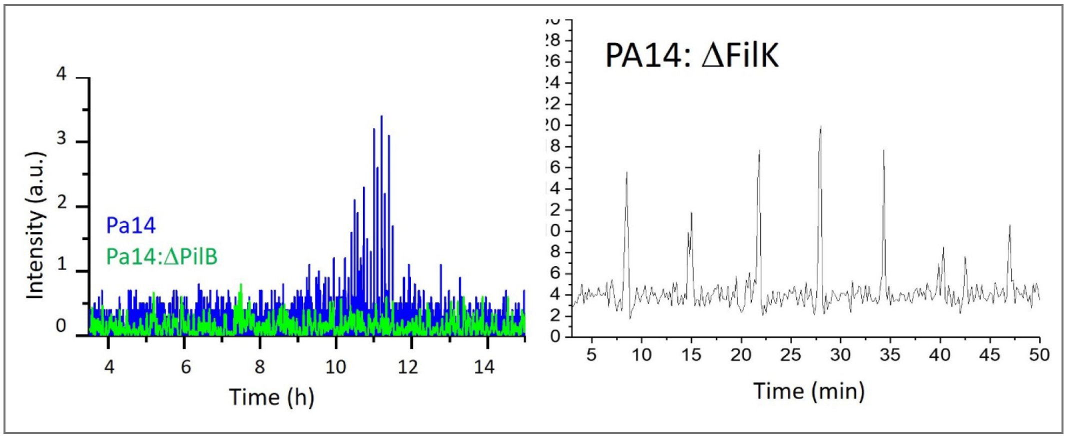


Supplementary Figure 3. **Image showing the elastic properties of the biofilm (PN) at the center of the colony.**

The biofilm was elastically stretched using a sharp needle. Unlike the finger-forming colony edge, this elastic central region generates spiral waves.

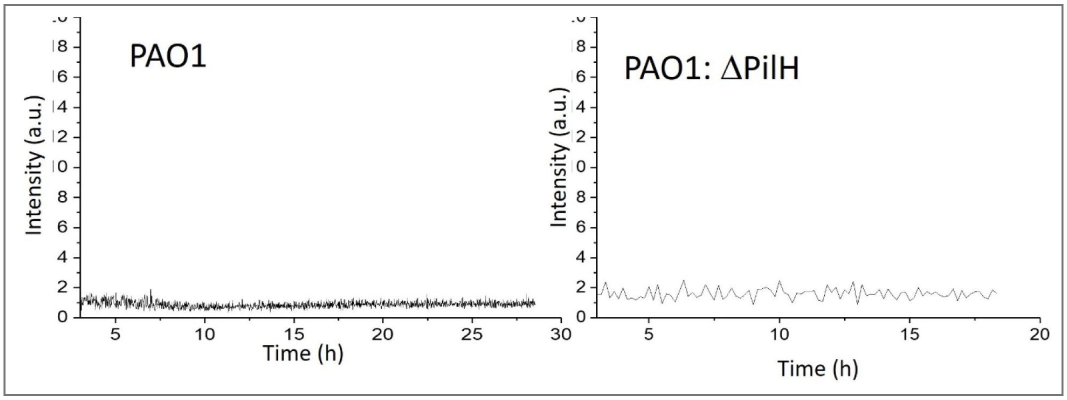
Supplementary Figure 4. **In PA14 strains, mutations in pilB and fliK were introduced to identify the processes responsible for driving the oscillations.**

The pilB mutation abolished wave formation, whereas wave emergence persisted in the fliK mutant, which lacks functional flagella.



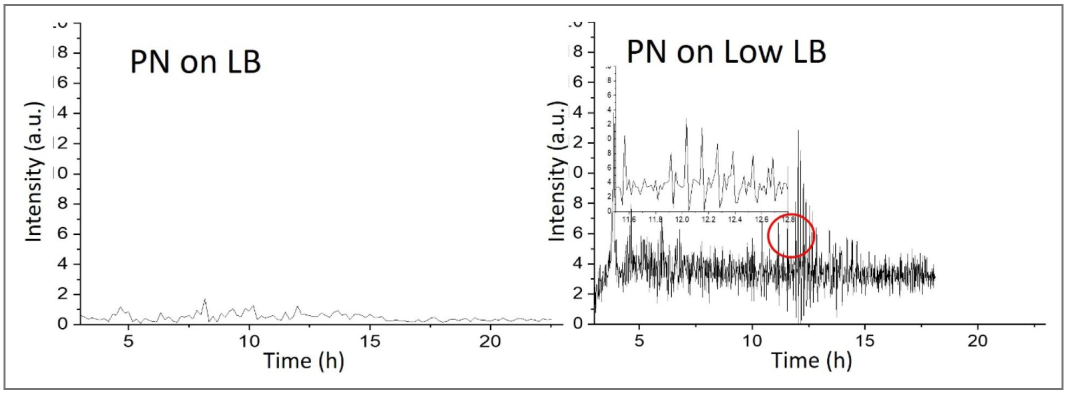
Supplementary Figure 5. **The PAO1 strain did not generate surface waves.**

Additionally, the hyperpiliated pilH mutant also failed to exhibit oscillatory wave behavior.



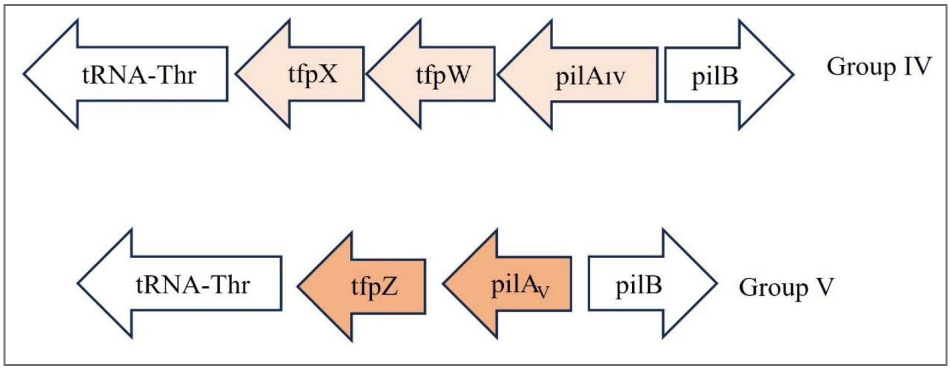
Supplementary Figure 6. **Regular LB plates do not provide sufficient conditions for wave generation in PN.**

However, eliminating yeast extract and replacing tryptone with a low concentration (0.2×) peptone restored wave formation.

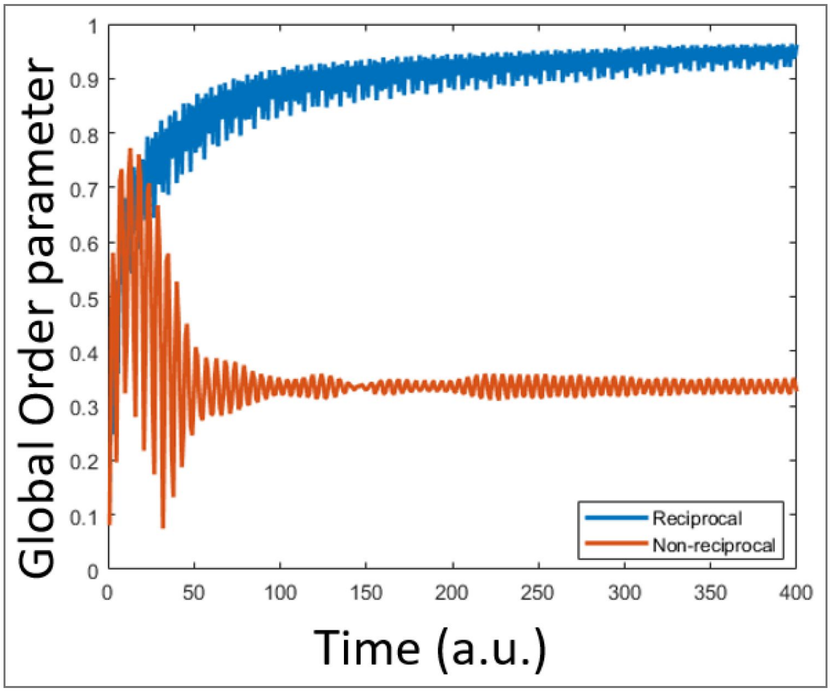


Supplementary Figure 7. Comparative analysis of the genomic region encoding pili subunit groups reveals that PA14 contains Group 3 pili, while PN exhibits a Group 4 and 5-like structure.

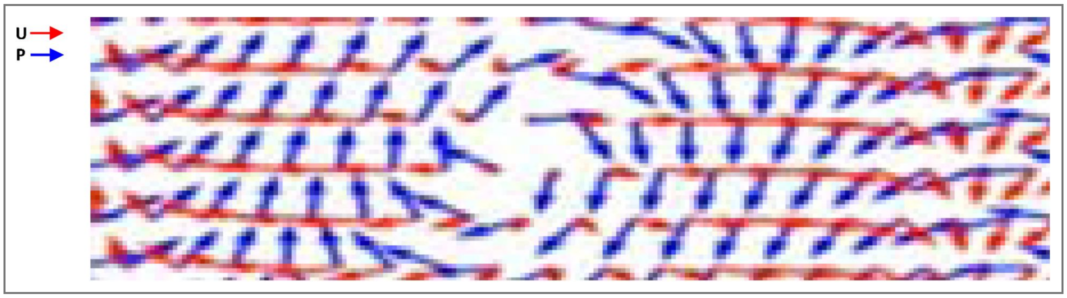
Both strains also encode accessory proteins associated with unknown pilus function.



Supplementary Figure 8. Global order parameter as a function of time for reciprocally and nonreciprocally coupled oscillators

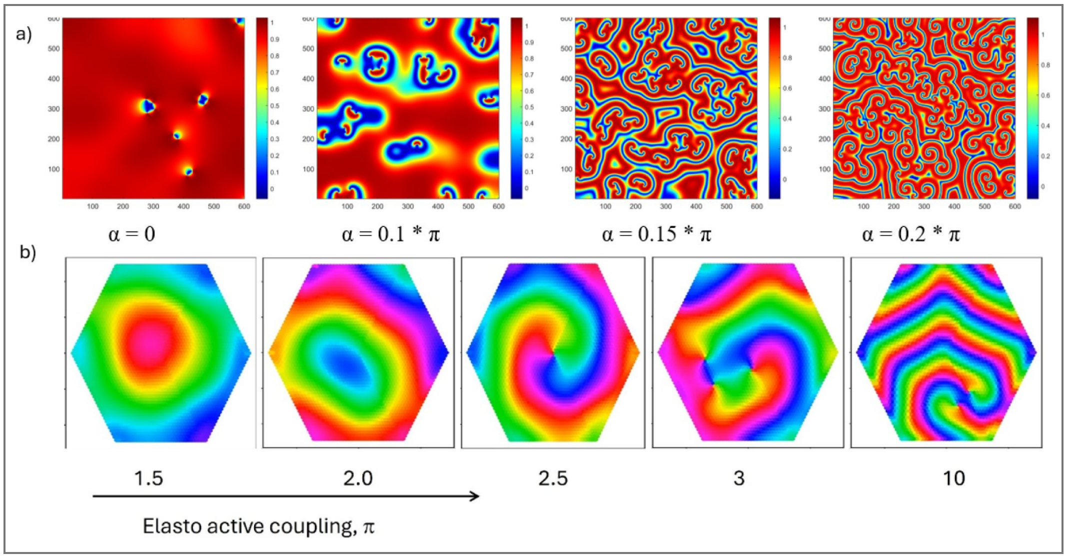


Supplementary Figure 9. Vector configuration around a topological defect, where the displacement vector changes direction.



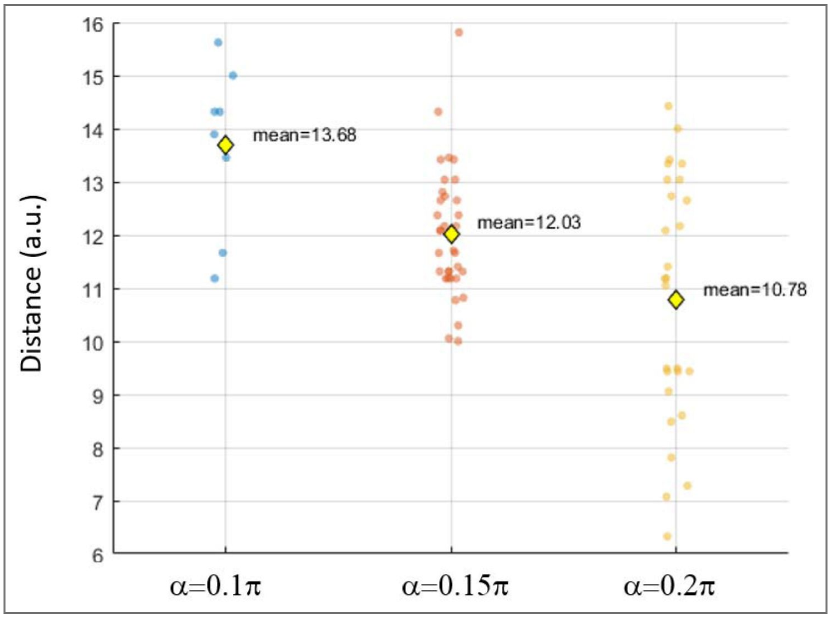
Supplementary Figure 10. Defect dynamics as a function of the control parameter.

(a) In the nonreciprocally coupled oscillator model, a large number of defects persist at high nonreciprocity, whereas at low nonreciprocity, oppositely charged (+/−) defect pairs annihilate. (b) In the active gel model, similarly, +/− defect pairs remain stable at high nonreciprocity, demonstrating consistent defect stabilization across both systems.

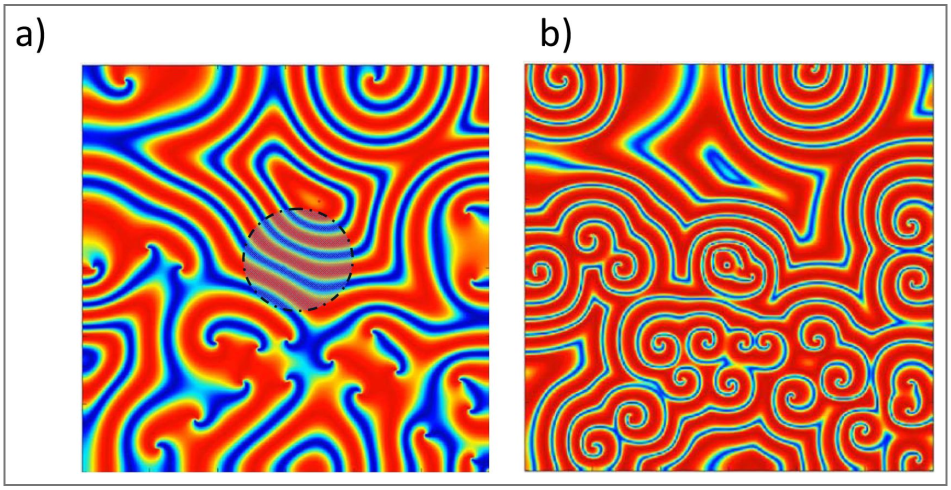


Supplementary Figure 11. Separation between the defect pairs.

The annihilation of +/− defect pairs, defect separation strongly depends on the nonreciprocity coefficient of the system. At high nonreciprocity, the system can sustain closely separated defect pairs. This regime corresponds to the dry condition of the biofilm.

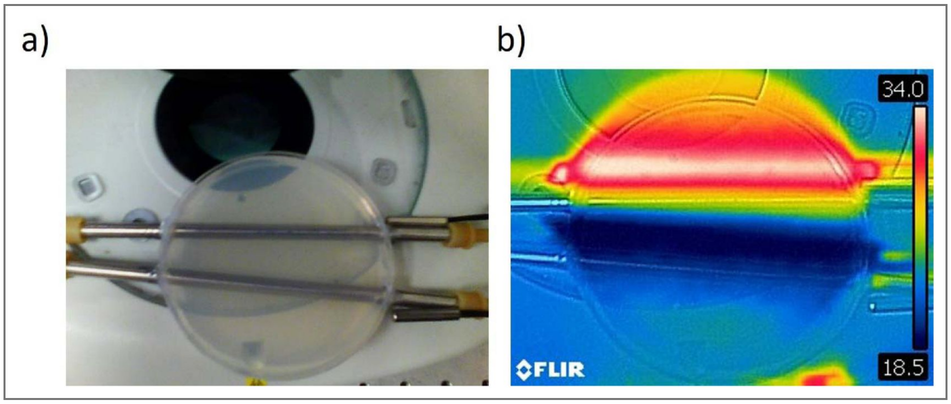


Supplementary Figure 12. **Simulation results of defect generation by introducing nonuniformities.**



Supplementary Figure 13. **Optical and thermal images of an NGM plate equipped with metallic pipes.**

A closed-loop water pumping-heating system was used to control the local temperature and create a temperature gradient between the pipes. The spacing between the pipes was varied to identify the optimal gradient profile.



Data availability

The critical experimental data generated or analyzed during this study are provided as supporting video files. We did not generate additional data sets. All the codes used in the study will beare available online (*https://github.com/akocabaslab/kuramoto-oscillators*).

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Additional information

Author contributions

B.A. and A.Ko. initiated the project and performed the experiments. B.A., Y.I.Y., and M.B. Y.G. developed and executed numerical modeling. B.K. conducted 16S DNA sequencing and GFP labeling of PN. N.G. performed genome sequence analysis. E.T.G. and A.Ü. developed and conducted imaging for the temperature gradient control system. S.K.Ö. and C.K. conceptualized non-Hermitian and PT-symmetry principles. A.Ko. and B.A. drafted the manuscript, with contributions from all authors toward the final manuscript.

Additional files

Video 1. Time-lapse imaging of active *Pseudomonas nitroreducens* biofilm surfaces generating metachronal waves. Global left-right symmetry is broken, and merging spiral waves at the edge drive unidirectional planar waves toward the biofilm center. The wave wavelength is approximately 100 µm. Associated with Figure 1a.

Video 2. Time-lapse imaging of circularly symmetric active biofilm surfaces generating inward-propagating metachronal waves. Associated with Figure 1b.

Video 3. High magnification (100X) time-lapse imaging of biofilm surfaces illustrating bacterial displacement and optical contrast changes.

Video 4. Time-lapse imaging of biofilm growth initiated from a single bacterium. After growth cessation, dense regions begin firing first, and waves dominate the biofilm surface.

Video 5. Time-lapse imaging of fingering formation at the biofilm edge. Bacteria remain motile and exhibit collective flow.

Video 6. Time-lapse imaging of the biofilm center generating propagating waves. Bacteria are attached to the surface; however, they periodically lift up and deform the surface, resembling Mexican-wave dynamics.

Video 7. Numerical simulation using the nonreciprocal Kuramoto model. Colors indicate oscillation phases. Spiral waves merge to form target waves, eventually converging into planar waves. Associated with Figure 2c.

Video 8. Numerical simulation based on the active solid model. Associated with Figure 2e.

Video 9. Time-lapse imaging of controlling the transition between waves to target and spiral by manipulating the moisture of biofilm surface. Associated with Figure 3d-g.

Video 10. Time-lapse imaging of a bacterial biofilm with broken left-right symmetry. Associated with Figure 6.

Video 11. Numerical simulation of propagating planar waves influenced by a frequency gradient. Associated with Figure 7c.

Video 12. Numerical simulation of defect dynamics triggered by nonuniformities at low nonreciprocal conditions (α=0.1π).

Video 13. Numerical simulation of defect generation triggered by nonuniformities at high (α=0.2π) nonreciprocal conditions.

Video 14. Time-lapse imaging of controlling propagating planar waves under a varying temperature gradient. Associated with Figure 7.

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72 **Hölscher T.,** et al. (2015) Motility, Chemotaxis and Aerotaxis Contribute to Competitiveness during Bacterial Pellicle Biofilm Development. *J Mol Biol* **427**:3695-3708 https://doi.org/10.1016/j.jmb.2015.06.014 | PubMed

Peer reviews

Reviewer #1 (Public review):

Summary:

Overall, this is an interesting paper. The authors identify several experimental knobs that can perturb mechanical wave behavior driven by pili feedback. They frame these effects in terms of nonreciprocal interactions. While nonreciprocity could indeed play a role, it raises the question of whether mechanical feedback might also contribute. Phenomenological models can be useful, but the model currently lack direct mechanistic insight. It would be more compelling to formulate the model around potential mechanochemical feedback, which could help clarify the underlying microscopic mechanisms.

Strengths:

Report of mechanical waves in bacterial collectives, mechanism has potential application in multicellular context such as morphogenesis.

Weaknesses:

A minor concern about the language of 'left-right asymmetry.' I believe the correct term is simply 'radial asymmetry' which is a distinct concept. Left-right is not well defined in the current context.

https://doi.org/10.7554/eLife.107609.2.sa2

Reviewer #3 (Public review):

Summary:

The revised manuscript presents a compelling study of radially propagating metachronal waves on the surface of Pseudomonas nitroreducens biofilms, combining experiments with two theoretical descriptions (a local phase-oscillator model and an active solid/active gel model). The central experimental findings-spiral/target/planar wave patterns, their controllability via water/PEG/temperature perturbations, and the correlation between frequency gradients and propagation direction-remain highly interesting and relevant to both bacterial biophysics and active-matter physics. The revised manuscript also adds substantial new material, including additional analyses of defect dynamics and clearer discussion of the relationship between the two models. The study continues to have a strong interdisciplinary appeal and the potential to stimulate further work on collective oscillations in biological active media.

Strengths:

The authors have substantially addressed the major conceptual issue raised in the previous round by clearly distinguishing between nonreciprocity and frequency gradients / global asymmetry. This clarification significantly improves the theoretical interpretation and resolves an important source of confusion in the original version.

The revised manuscript also improves the connection between the phase-oscillator and active-solid descriptions. In particular, the authors now explain more explicitly how the phase variable is defined in the reduced oscillatory dynamics of confined biofilm motion, and they state that they added a schematic illustration and simulation details (including parameter values and the elastic-force definition) to improve reproducibility. This directly addresses one of my previous major concerns.

A notable improvement is the newly added defect-based analysis of waveform transitions (spiral -> target -> planar). The revised text argues that defect motility is a key control parameter, linked experimentally to moisture-dependent elasticity and theoretically to nonreciprocity / defect-pair stability. This provides a more concrete mechanistic bridge between experimental perturbations and the modeling framework than in the previous version.

The manuscript now gives a clearer experimental-theoretical narrative for how environmental manipulations (drying, water addition, PEG, heating) affect wave patterns through changes in effective elasticity and activity, including a useful distinction between short-timescale and long-timescale temperature effects. This added discussion strengthens the biological interpretation and makes the modeling assumptions easier to follow.

Weaknesses:

The main remaining limitation is the level of quantitative correspondence between theory and experiment. The revised manuscript now provides a stronger qualitative/mechanistic link, but the mapping between model parameters (e.g., effective coupling terms / elasto-active parameters) and directly measurable biofilm properties is still limited. The authors acknowledge this point, and I agree that it is technically challenging in the present system. However, this means the theoretical framework is currently most convincing as an effective mechanistic model rather than a quantitatively predictive one.

Relatedly, some conclusions about parameter-level control (especially in connecting moisture/temperature manipulations to specific model parameters) remain qualitative. I do not view this as fatal, but I recommend that the manuscript clearly state this scope and avoid overstating the quantitative predictive power of the theory.

Although the terminology has improved compared with the original version, the revised manuscript still uses "left-right asymmetry" in places where the underlying geometry and symmetry are more general (e.g., radial inward propagation in circular colonies). Since this wording was one of the original points of confusion, I suggest one final pass to ensure the symmetry language is consistently precise throughout the main text and figure captions.

https://doi.org/10.7554/eLife.107609.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***eLife Assessment***
>
> *This important study concerns the propagation of waves in bacterial biofilms, bridging active matter physics and bacterial biophysics. While the experimental observations are solid, the theoretical interpretation and model validation are currently incomplete and require further refinement. This work will be of interest to microbiologists, biophysicists, and researchers studying collective behavior in biological systems.*

In the revised manuscript, we have added new experimental results that strengthen the connection between our observations and the modeling framework used to interpret the collective oscillations. We have not introduced a new theoretical model; rather, we employed established active matter models and sought to link the observed phenomena to these frameworks. In particular, our new data demonstrate that the transition between the motile and biofilm-forming states specifically modulates the elasticity and elasto active coupling of the bacterial structure. This behavior is in excellent agreement with the predictions of the active solid model. All the experimental details are given below. We believe that the revised version of the manuscript now establishes this connection more clearly and convincingly.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *Overall, this is an interesting paper. The authors have found multiple experimental knobs to perturb a mechanical wave behavior driven by pilli feedback. The authors framed this as nonreciprocal interactions - while I can see how nonreciprocity could play a role - what about mechanical feedback? Phenomenological models are fine, but a lack of mechanistic understanding is a weakness. I think it will be more interesting to frame the model based on potential mechanochemical feedback to understand microscopic mechanisms. Regardless, more can be done to better constrain the model through finding knobs to explain experimental observations (in Figures 3, 4, 5, and 7).*

We thank the reviewer for the positive assessment and for highlighting this important point. The reviewer is correct that the phenomenological Kuramoto-based model does not explicitly show the detailed cell–cell interactions. However, the active solid model is formulated on detailed elastic couplings and active forces, which inherently represent mechanical feedback within the biofilm structure. In this framework, nonreciprocity emerges naturally from the tensorial nature of active forces between bacteria—a concept already well established in the active matter literature. Importantly, this mechanism is purely mechanical and closely parallels nonreciprocal hydrodynamic interactions among active particles, which also arise from tensorial couplings.

In our system, elastic interactions within the biofilm matrix, combined with pilus-generated active forces, provide a natural origin for nonreciprocal interactions. To further validate this, we improved our imaging to record single-cell dynamics both at the colony edge and on the biofilm surface. (new supplementary Video). These experiments show that motile bacteria at the leading edge of the biofilm structure do not generate waves, whereas stationary bacteria within the biofilm display local oscillations within the elastic network. This observation supports the view that collective oscillations are a property of the elastic biofilm state rather than of freely motile cells.

Moreover, the main control parameter for these oscillations is the ratio between elastic strength and the active force generated by pili. In the active solid model, this ratio is captured by the parameter π and alpha terms. Experimentally, we can tune this ratio simply by adding or removing water from the biofilm, thereby modulating its elasto active coupling. We further motivated the controllability of this feature experimentally. We let the plate dry nonuniformly and observed that the transition between spiral target and plane waves could emerge spontaneously across the plate (see Figure 3a). This observation also states the importance of moisture in the biofilm. Starting from this point we established the connection between experimental observation and modelling. In our new simulations we also noticed that the transition from spiral to target wave is particularly driven by merging processes of different topological charges +/- 1 spiral pairs. This critical point was also confirmed by modelling which links the process to elasto active coupling. Further we supported our claim by imagining the edge and the biofilm structure. These new results clarify that elastic structure of the biofilm is critically important (Supplementary Figure 3). We have clarified this mechanistic link in the revised manuscript and rewritten the relevant sections to make this connection explicit.

Modification in the manuscript:

“To gain deeper insight into the mechanisms underlying wave formation, we imaged the dynamics of individual bacteria from the fingering regions toward the center of the biofilm. This distinction is critical because, unlike the biofilm center, the edges do not generate waves. We observed that bacteria near the fingering regions remain motile and exhibit collective flow. In contrast, bacteria at the biofilm center are surface-attached and undergo periodic lifting motions. This behavior strongly resembles Mexican-wave dynamics.”

“We further found that the central region of the biofilm is mechanically more elastic, whereas the edge regions—where wave formation is absent—are motile. These observations suggest that gradual biofilm maturation is a key factor that transforms motile bacteria into a periodically moving but spatially constrained state. Consistent with this picture, the PAO1 strain, which has a strong biofilm-forming capability, completely suppresses surface oscillations. In contrast, the PA14 strain exhibits intermediate behavior, sustaining a partial transition between motile and locally constrained dynamics. Remarkably, signatures of this transition and wave generation are already detectable at the earliest stages of finger formation.”

> *Strengths:*
>
> *The report of mechanical waves in bacterial collectives. The mechanism has potential application in a multicellular context, such as morphogenesis.*

We thank the reviewer for the positive assessment and for highlighting this potential broad impact of our findings.

> *Weaknesses:*

> *My most serious concern is about left-right symmetry breaking. I fail to see how the data in Figure 6 shows LR symmetry breaking. All they show is in-out directionality, which is a boundary condition. LR SM means breaking of mirror symmetry - the pattern cannot be superimposed on its mirror image using only rigid body transformations (translation and rotation) - as far as I am aware, this condition is not satisfied in this pattern-forming system.*

We thank the reviewer for pointing out this critical issue. We acknowledge that we overlooked the distinction between biological and physical definitions of left–right symmetry in our initial submission, and we agree that our terminology was confusing.

In developmental biology, the term “left–right symmetry breaking” is often used to describe asymmetric flows generated by nodal cilia, which subsequently establish developmental asymmetry. This usage differs fundamentally from the physical definition of mirror symmetry breaking, which refers to chirality switching upon mirror reflection. As the reviewer correctly noted, our system does not exhibit mirror symmetry breaking in this strict physical sense.

To avoid confusion, we have revised the manuscript and replaced the term left–right symmetry breaking with left–right asymmetry between the edge and the center of the biofilm. This asymmetry arises from frequency gradients across the biofilm and is not a trivial boundary effect. For circular colonies, this phenomenon is more accurately described as radial asymmetry. We have rewritten the relevant sections of the manuscript to clarify this distinction and prevent misinterpretation.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *This manuscript by Altin et al. examines the dynamics of bacterial assemblies, building on previously published work documenting mechanical spiral waves. The authors show that the emergent dynamics can be influenced by various factors, including the strain of bacteria and water content in the sample. While the topic of this paper would be of broad interest, and the preliminary results are certainly interesting, various aspects of this paper are underdeveloped and require further exploration.*
>
> *Strengths:*
>
> *One of the nice features of this system is the ability to transition between the different states based on the addition or withdrawal of water. The authors use a similar experimental model system and mathematical model to previously published work (Reference 49), but extend by showing that the behaviour can be modified through simple interventions. Specifically, the authors show that adding water droplets or drying the sample through heating can result in changes in the observed wave structure. This represents a possible way of controlling active matter.*
>
> *The mathematical model proposed in this paper involves a phase-oscillator model of Kuramotostyle coupling (similar to previously reported models). A non-reciprocal phase lag is introduced in order to facilitate the patterns seen in experiments. The qualitative agreement in the behaviour is quite striking, showing both spiral waves and travelling waves.*

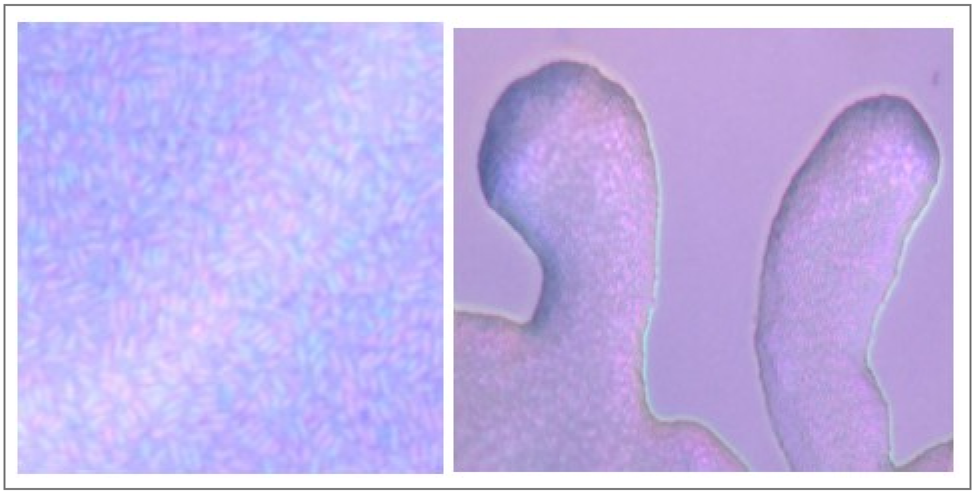
We thank the reviewer for the positive assessment and for pointing out areas that required further development. The reviewer is correct that our work builds on previously reported bacterial spiral wave systems; however, there are several significant differences that we now emphasize more clearly in the revised manuscript.

First, our study involves a different bacterial species and reveals a distinct dynamical process: the waves we report are strictly localized on the surface of the biofilm, in contrast to the bulk oscillations detected through density fluctuations in the earlier work (Ref. 49). The surface waves in our system resemble “Mexican wave”-like motions, in which surface bacteria periodically lift upward. To highlight this key distinction, we performed new imaging experiments that directly visualize this process. (New Video 5 and 6, Author response image 1).

Second, we systematically compared different bacterial strains, including pathogenic species such as *P. aeruginosa* PA14 and PAO1, alongside our BSL-1 strain. This comparative approach demonstrates that the observed phenomenon spans strains with different pathogenicity levels, and genetic variations while also showing that our strain provides a safer and more broadly usable model system for laboratory investigations.

Third, the modeling frameworks differ. Whereas the referred study relied primarily on phase models similar to those used in cilia systems, we combine a delayed Kuramoto-style oscillator model with an active solid model. This combination provides both a phenomenological description and a physical interpretation of the collective dynamics. We acknowledge that, in the original submission, the physical interpretation of the model in relation to our experimental system was underdeveloped. In the revision, we have now established this link explicitly through the elasticity and elasto active coupling of the biofilm. Specifically, we show that the transition from motile to biofilm states is accompanied by changes in elasticity, which directly influence the observed transitions between different types of wave defects. This connection is consistent with prior theoretical works and has even been only studied in robotic active matter systems.

Together, these clarifications and new results reinforce the novelty of our findings and establish a stronger connection between the experiments and the modeling framework.



Author response image 1. Comparison between the elastic biofilm core and the motile colony edge.
Highresolution video recordings revealing individual bacterial motion highlight the key physical differences driving wave-generating. Time-lapse snapshots show that bacteria at the colony edge move freely and form fingering structures, whereas bacteria in the elastic central biofilm periodically lift vertically, producing a Mexican-wave–like collective motion across the surface. See new Video

> *Weaknesses:*
>
> *The principal observation of the paper - that spiral waves emerge in these systems and can be controlled in various ways - is not linked to microscale dynamics at the cell level. It*

> *is recognised that hydrodynamics can introduce non-reciprocity, an essential ingredient of this model. However, in this work the authors have not identified a physical mechanism for the lag, e.g., either through steric interactions or hydrodynamic disturbances. This is also relevant in the phase oscillator modelling section. In low Reynolds number flows, dynamics are instantaneously determined. In this light, what does the phase lag term represent?*

The reviewer is correct that, at low Reynolds numbers, fluid dynamics are instantaneous and do not generate real temporal delays. However, nonreciprocity in hydrodynamic interactions can still emerge from the tensorial structure of the Blake–Oseen Green's function. In this formalism, the effective asymmetry can be represented mathematically as a phase-lag–like term. This has been theoretically demonstrated in Ref.40. While this is not a literal time delay, it functions analogously by breaking odd symmetry in the coupling.

In our system, strong long-range hydrodynamic interactions are absent, as the bacteria are embedded in an elastic biofilm matrix. Instead, the dominant interactions are active elastic couplings mediated by pili and biofilm structure. The elastic solid model behaves in a way that is conceptually similar to the hydrodynamic case: pili-induced deformations of the elastic medium produce anisotropic stresses that play a role analogous to the tensorial hydrodynamic Green's function. Thus, the phase-lag term in our Kuramoto-based model can be interpreted as an effective representation of these nonreciprocal elastic interactions.

We have clarified this point in the revised manuscript by explicitly connecting the phenomenological phase-lag term to the underlying elastic coupling in biofilms.

> *What is the origin of the coupling term, b? Can this be varied systematically or derived from experimental measurements or parameters?*

The term b represents the enhanced elasto-active coupling of the pili process. The length of the Pili varies, and the elongated Pili has more potential to modulate the coupling between bacteria which is known to depend on a critical threshold. This process resembles the pinning dynamics and is driven by the activity of molecular motors within the pili machinery. However, the detailed mechanisms that set the effective coupling strength remain highly complex and are not yet fully understood.

At present, we do not have a direct way to systematically manipulate b in experiments. A major technical limitation is the nanoscale nature of type IV pili: these protein assemblies are extremely small and difficult to monitor or manipulate directly. Even basic tools such as GFP-based labeling have proven challenging to implement, which restricts our ability to track the detailed dynamics of these structures in live biofilms.

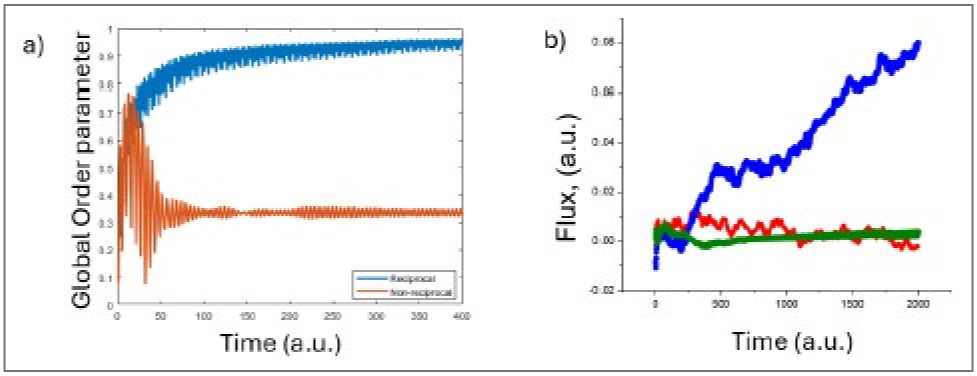
While we cannot currently derive b directly from experimental parameters, we emphasize in the revised manuscript that b should be understood as an effective parameter capturing the excitability of pili retractions. We also highlight this limitation and note that future advances in molecular imaging and manipulation of pili will be essential for quantitatively linking b to microscopic processes.

> *Classification of wave properties is an important aspect of this paper, but is not accomplished in a quantitative sense. What is the method for distinguishing between travelling and spiral waves? There is a range of quantitative tools that could be used to investigate these dynamics (and also compare quantitatively with the models). For example, examining the correlation functions and order parameters could assist with the extraction of wave features (see extensive literature on oscillator models).*

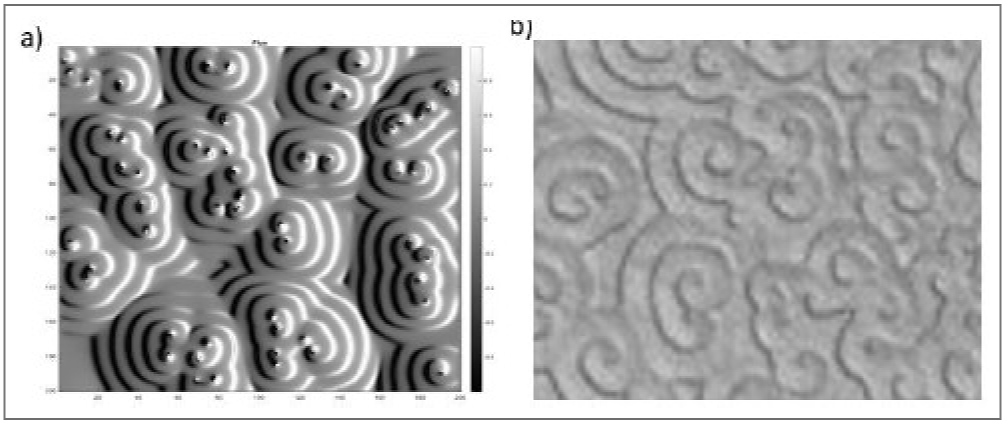
We thank the reviewer for emphasizing this important point. In the revised manuscript, we have incorporated the classic Kuramoto order parameter (S) to characterize the dynamics in our model simulations. However, this metric is not directly applicable to our experimental system, because we cannot resolve the phase of individual bacteria at large scales.

Instead, we have focused on a flux-based parameter, as previously used in Ref. 40, which can be measured experimentally from collective surface dynamics. Interestingly, we find that the directional flux extracted from our experimental movies closely matches the trends predicted by the model order parameter. We suspect that this similarity arises from the combination of our optical illumination method and the characteristic surface modulations of the biofilm. While we currently lack a rigorous theoretical justification for this correspondence, so we want to keep this discussion in the review document.

In summary, we now use the classic Kuramoto order parameter in simulations and rely on the experimentally accessible flux measure for our experimental data. This dual approach allows us to compare model and experiment in a consistent manner.



Author response image 2. Critical order parameters of the coupled biofilm system. (a) The Kuramoto global order parameter increases continuously as the system becomes globally synchronized. In contrast, in the nonreciprocally coupled system the order parameter saturates at a critical level. (b) In the experimentally observed biofilm, however the flux generated by the coupled oscillations provides a more appropriate measure of synchronization. Blue curves indicate directionally propagating planar waves, red curves correspond to spiral wave formation, and green curves represent the globally synchronized reciprocal system.



Author response image 3. Comparison of flux profiles of the simulations with experimental measurements. Directional optical illumination enhances the flux term on the surface of the biofilm.

> *The methodology of changing the dynamics through moisture content appears to be slightly underdeveloped, e.g., adding water involves a droplet, and removing water is accomplished by heating (which presumably could cause other effects). Could the dynamics not be controlled more directly by varying the humidity?*

We thank the reviewer for this valuable suggestion. Our results indicate that water content in the biofilm plays a key role in driving the transition to the biofilm state by modulating its elasticity. During the initial submission, we did not know how to systematically vary humidity without simultaneously altering temperature. Standard approaches typically involve water evaporation in controlled chambers, which inherently changes both parameters.

Following the reviewer's recommendation, we first measured the ambient moisture levels inside closed culture plates. To our surprise, the relative humidity was already ~98%, leaving virtually no room to increase it further. We then attempted to decrease humidity by flowing dry synthetic air, but even under these conditions we could not reduce it below ~85%, and achieving this required unrealistically high flow rates. Moreover, we noticed that in closed-lid NGM plates, evaporation is already substantial, and when the lid is left open the evaporation rate reaches ~1 µm/s. This rapid surface thinning severely limits the quality of long-term time-lapse imaging.

Taken together, these technical constraints explain why we have to reliy on localized perturbations such as water droplets and heating rather than global humidity control. We have clarified this point in the revised manuscript and now explicitly discuss both the challenges and limitations of humidity-based approaches.

> *At the same time, the authors also mention that temperature itself plays a role in shaping the behaviour. What is the mechanism for this? Is it just through evaporation? Since the frequency increases with temperature, could it just be that activity increases with temperature?*

We thank the reviewer for raising this critical point. We believe that temperature has two distinct impacts operating on different timescales.

Short timescale (~minutes): We observed that biofilm oscillations respond to temperature changes very rapidly and in a reversible manner. This timescale is too short to be explained by modulation of water content or bulk elasticity of the biofilm. Instead, we attribute the immediate frequency increase to enhanced biological activity of the bacteria at elevated temperatures.

Long timescale (~tens of minutes to hours): During processes such as the transition from planar to spiral waves, prolonged heating can significantly alter the biofilm structure. These changes are not reversible and likely involve modifications of elasticity and other structural properties.

In the modeling framework, the short-timescale effect is represented as an increase in the active force term, while the long-timescale effect is captured by concurrent changes in both the active force and the elastic properties of the biofilm. We have clarified this mechanism and its representation in the revised manuscript.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *This manuscript presents a novel investigation into unidirectionally propagating waves observed on the surface of Pseudomonas nitroreducens bacterial biofilms. The authors explore how these waves, initially spiral in form, transition into combinations of spiral,*

> *target, and planar patterns. The study identifies the periodic extension-retraction cycles of type IV pili as the driving mechanism for wave propagation, which preferentially moves from the colony's edge to its center. Furthermore, the manuscript proposes two theoretical models-a phase-oscillator model and a continuum active solid model-to reproduce these phenomena, and demonstrates how external manipulations (e.g., water droplets, temperature, PEG) can control wave patterns and direction, often correlating with oscillation frequency gradients. The work aims to bridge the fields of activematter physics and bacterial biophysics by providing both experimental observations and theoretical frameworks for understanding these complex biological wave phenomena.*

We thank the reviewer for the positive assessment of our work and for highlighting both the novelty and the key contributions of our study.

> *Strengths:*
>
> *The experimental discovery of unidirectionally propagating waves on bacterial biofilms is highly intriguing and represents a significant contribution to both microbiology and active-matter physics.*
>
> *The detailed observations of wave pattern transitions (spiral to target to planar) and their response to various environmental perturbations (water, temperature, PEG) provide valuable empirical data. The identification of type IV pili as the driving force offers a concrete biological mechanism. The observed correlation between frequency gradients and wave direction is a compelling finding with potential for broader implications in understanding biological pattern formation. This work has the potential to stimulate further research in the collective behavior of living systems and the physical principles underlying biological organization.*

We thank the reviewer once again for emphasizing the importance of wave directionality. We also believe that this phenomenon may provide insight into early symmetry-breaking processes observed in developmental biology, where oxygen or nutrient gradients in dense environments could play a similar role.

> *Weaknesses:*
>
> *The manuscript attempts to link unidirectional wave propagation to non-reciprocal couplings but ultimately shows that the wave direction is determined by the gradient of the oscillation frequency. The couplings in the two theoretical models are both isotropic and thus cannot dictate the wave direction. A clear distinction should be made between non-reciprocity as a source of wave generation and non-uniformity as a controlling factor of wave direction.*

We greatly appreciate the reviewer's careful evaluation, particularly for highlighting this important and often confusing distinction. The relationship between nonreciprocity, spontaneous symmetry breaking, and frequency gradients has also been a challenging concept for us and required significant effort to clarify.

Recent theoretical studies have established that traveling wave formation requires nonreciprocity, which provides a framework for understanding phenomena ranging from spiral to target and planar waves. In our system, nonreciprocity arises between the displacement field (U) and the pili force vector (P): as a result in broken phase U effectively "chases" P, breaking PT symmetry locally and thereby enabling the generation of local directional flux and traveling waves. In this sense, nonreciprocity is essential for travelling wave generation and spontaneous symmetry breaking in either direction.

However, we now agree that global directionality (always from right to left, or edge to center) is set by an independent factor—namely, the oscillation frequency gradient across the biofilm. Thus, while nonreciprocity determines whether waves can travel, frequency gradients determine the large-scale direction in which they propagate. Put differently, PT symmetry is already broken spiral waves due to nonreciprocity, but global asymmetry (frequency gradients) is required to align the overall propagation in one direction.

We have clarified this distinction in the revised manuscript, emphasizing that nonreciprocity is a necessary ingredient for travelling wave generation, whereas global asymmetry controls global wave direction.

Modification in the manuscript:

"We should note that traveling waves indicate broken PT symmetry between these fields triggered by nonreciprocity, with spiral waves serving as a classic signature of this phenomenon. A further transition from spiral to planar waves reflects an overall asymmetry in the frequency profile, which is not directly related to PT-symmetry breaking."

> *The relationship between the phase oscillator model and the active solid model is unclear. Given that U and P are both dynamical variables evolving in three-dimensional space, defining the phase Φ precisely in the phase space spanned by U and P could be challenging. A graphical illustration of the definition of Φ would be beneficial. To ensure reproducibility of the numerical results, the parameter values used in the numerical simulations and an explicit definition of the elastic force in the active solid model should be provided.*

We agree with the reviewer that the relationship between the phase oscillator model and the active solid model can be confusing, but establishing this link is essential to connect different modeling approaches in the literature. As the reviewer notes, in a fully three-dimensional setting with freely moving bacteria, defining the oscillation phase (Φ) in the phase space spanned by U and P is indeed complicated.

However, our recent imaging results show that bacteria within the biofilm do not undergo large translational motions but instead exhibit periodic "Mexican wave"-like oscillations. These oscillations are confined to a restricted phase space, which allows us to define Φ in a straightforward way. In this context, the phase oscillator model becomes a natural reduction of the dynamics.

Similarly, in the active solid (or active gel) model, we can plot not only the displacement and force vectors but also the local phase, which shows strong agreement with the phenomenological Kuramoto-style model. To make this connection clearer, we have now included a schematic illustration in the revised manuscript that explicitly shows how Φ is defined in the reduced phase space, and we provide the parameter values used in the simulations as well as the explicit definition of the elastic force in the active solid model to ensure reproducibility.

> *The link between the theoretical models and experimental results is weak. For example, the propagation of the kink from the lower to the higher part of the surface (Figure 1e) could be addressed within the framework of the active solid model. The mechanism of transition from spiral to target waves (Figure 3a), b)) requires clarification, identifying which model parameter is crucial for inducing this transition. The wave propagation toward the lower frequency side is numerically demonstrated using the phase oscillator model, but a physical or intuitive explanation for this phenomenon is missing. Also, the wave transitions induced by the addition of water droplets and temperature rise are not linked to specific parameters in the theoretical models.*

We thank the reviewer for highlighting this important weakness, which was also consistently noted by the other reviewers. We fully agree that the link between our theoretical models and experimental results required significant strengthening.

With improved imaging in the revised study, we were able to uncover additional connections that help establish this link more clearly. We acknowledge that our ability to measure detailed biofilm parameters is limited, which restricts us from providing fully quantitative mappings. Nonetheless, based on the reviewers' suggestions, we carried out additional imaging and simulations to compare bacterial dynamics at the colony edge and within the biofilm surface. These data confirm that cells within the biofilm undergo restricted, “Mexican wave”-like oscillations, emphasizing the critical role of elasticity in governing the collective dynamics.

Experimentally, we found that adding water or PEG, or alternatively inducing drying, strongly modulates the effective elasticity of the biofilm. Within the active solid framework, elasticity and the elasto-active coupling are the key parameters controlling the system. By tuning these parameters in simulations, we could reproduce the qualitative transitions observed experimentally. Specifically, we observed that:

At low elasticity, topological defects are mobile and can move, merge, or annihilate, leading to the emergence of planar waves.

At high elasticity, defects remain pinned, across the biofilm surface, dominating the dynamics.

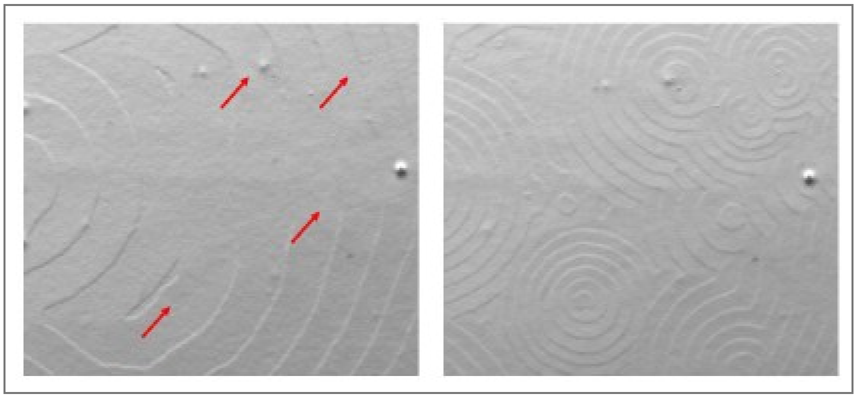
These observations suggest that the motility of defects is the crucial parameter governing the transition between spiral, target, and planar waves. Although we cannot independently manipulate each parameter in experiments, varying the moisture content provides an effective and experimentally accessible control.

Finally, our simulations and new analyses reveal that spiral defect cores can move and merge to form target waves or annihilate entirely—processes that we also observe experimentally. This rich dynamical behavior underscores the importance of elasticity in shaping pattern transitions, and we believe it warrants further theoretical exploration. We have clarified this connection and its implications in the revised manuscript.

First, we compare defect dynamics in both Kuramoto-based simulations and the active solid model. Both systems exhibit similar defect-survival behavior. As shown in the review , pairs of unlike (+/−) defects can stably persist only at high nonreciprocity. We further quantify this behavior by plotting the separation distances between unlike defect pairs and find that short-range defect separations are possible exclusively in the high-nonreciprocity regime Supplementary Figure 11.

This high-nonreciprocity regime corresponds to the dry biofilm state. Increasing moisture reduces elasticity, leading to the loss of stable defect dynamics and promoting the annihilation of unlike defect pairs, which in turn drives the system toward target-wave formation and ultimately planar waves. Conversely, heating the biofilm removes water, enhances elasticity, and increases the system's ability to sustain closely separated defect pairs.

Experimentally, we further observe that removing water by heating enhances surface nonuniformities, which readily trigger defect-pair formation. To investigate this mechanism, we performed additional simulations in which local nonuniformities were introduced Supplementary Figure 12. Consistent with experiments, defect-pair generation occurs only at high nonreciprocity, where pairs of unlike defects can be stably maintained. Experimental observation (Author response image 4) also show that surface nonuniformities on the biofilm surface similarly trigger the formation of closely separated defect pairs. We have updated the details of the defect dynamics in the revised manuscript to clarify the transition between these waves.



Author response image 4. Experimental observation showing that small surface nonuniformities on the biofilm surface trigger the formation of closely separated defect pairs. Arrows indicate the position of the nonuniformities

Modification in the manuscript:

Defect dynamics controlling the transition between spiral to target waves

“To better understand the dynamics of the transition between different form of the waves we focused on numerical simulations. We noticed that the motility of defects is the crucial parameter governing the transition between spiral, target, and planar waves varying the moisture content provides an effective and experimentally accessible control this motility. Our analyses revealed that spiral defect cores can move and merge to form target waves or annihilate entirely—processes that we also observe experimentally. This rich dynamical behavior underscores the importance of elasticity in shaping pattern transitions. First, we compare defect dynamics in both Kuramotobased simulations and the active solid model. Both systems exhibit similar defect-survival behavior. As shown in Supplementary Figure10, pairs of unlike (+/−) defects can stably persist only at high nonreciprocity. We further quantify this behavior by plotting the separation distances between unlike defect pairs and find that short-range defect separations are possible exclusively in the high-nonreciprocity regime (Supplementary Figure11). This high-nonreciprocity regime corresponds to the dry biofilm state. Increasing moisture reduces elasticity, leading to the loss of stable defect dynamics and promoting the annihilation of unlike defect pairs, which in turn drives the system toward target-wave formation and ultimately planar waves. Conversely, heating the biofilm removes water, enhances elasticity, and increases the system’s ability to sustain closely separated defect pairs. Experimentally, we further observe that removing water by heating enhances surface nonuniformities, which readily trigger defect-pair formation (Supplementary Video9). To investigate this mechanism, we performed additional simulations in which local nonuniformities were introduced (Supplementary Video12-13). Consistent with experiments, defect-pair generation occurs only at high nonreciprocity, where pairs of unlike defects can be stably maintained. Experimental observation (Supplementary Video9) also show that surface nonuniformities on the biofilm surface similarly trigger the formation of closely separated defect pairs.”

All the recommended points have been addressed in the revised manuscript.

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