Type IV Pilus retraction forces shape *Neisseria gonorrhoeae’s* early biofilm physiology

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### Abstract

The mechanics of the extracellular bacterial appendage, type IV pilus (tfp), mediates many functions, such as cellular twitching motility, aggregation, and DNA transformation. The role of mechanical cues in signaling pathways is often confounded by a myriad of other cellular and environmental factors. Here, we aim to assess the relationship between tfp activity and its roles in broader physiological functions: survival, antibiotic susceptibility, and gene expression. We measured the differential survival rate between *Ng* wild-type (WT) and a tfp retraction-deficient mutant (Δ*pilT::Kan*), and found that survival of Δ*pilT::Kan* is rescued to WT rates when mixed with WT bacteria at equal titers. When tfp engagement is modified, we measure changes in antibiotic susceptibility of bacterial aggregates (microcolonies) that correlates with tfp retraction engagement time. Survival rates are impacted when colonies are exposed to external global vibration stress. The *Ng* microcolonies gene expression spatiotemporal patterns are also disrupted on vibrating platforms, which suggests a global mechanosensitive mechanism. Collectively, these results suggest that mechanical retraction of tfp influences physiology through a pathway that still needs to be uncovered.

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Introduction

Within the last few decades, the fact that physical forces can dictate diverse biological outcomes from cell division to tumor development has become well established. The correct functioning of eukaryotic multicellular organisms requires complex genetic programs coupling chemical and mechanical cues. While most of the studied examples have featured eukaryotic systems, there is a growing awareness that physical forces also play an important role in the prokaryotic world. The bulk of our knowledge about bacterial physiology stems from the study of bacteria in suspension when we appreciate now that bacteria are more often part of dense mechanically connected communities called biofilms. The current study focuses on the role of mechanical forces during early stage biofilm formation in the causative agent of gonorrhea, *Neisseria gonorrhoeae*.

The mechanical nature of *Neisseria gonorrhoeae’s* extracellular appendage, the type IV pilus (tfp), makes for an ideal model to study the role of mechanical force in bacterial physiology. Tfp dynamics is orchestrated by a multimeric molecular machinery composed of approximately 15 proteins (Goosens et al. 2017). The main tfp protein filament is composed of a main subunit, PilE. These PilE monomers polymerize and extend into extracellular space by the AAA-ATPase, PilF. Pilus retracts into the cell due to the depolymerization of PilE, which is fueled by AAA-ATPase, PilT, from within the periplasmic membrane. PilT retracts a single pili and bundles of several pili in unison. Single pili retract 100 - 180 pN, while pili bundles can amount to forces up to 1 nN, 100,000 times stronger than the cell's own weight, which makes *Ng* the strongest microbe measured to date (Merz, So, and Sheetz 2000; Maier et al. 2002; Biais et al. 2008).

Tfp is essential for microcolony formation, and other versatile functions, like DNA horizontal transfer and twitching motility (Beatson et al. 2002; Berry and Pelicic 2015; Craig, Forest, and Maier 2019). Tfp performs these tasks by a repetitive “cast and pull” mechanism (Figure 1A) using dynamic extracellular adhesion filament from the cell body (Higashi et al. 2007). Microcolonies assemble into spherical ~20µm in diameter aggregates within 3 hours from single cells interacting with a surface, whether abiotic or biotic (Higashi et al. 2007). The only requirement for microcolony formation is through the physical intracellular interaction of their ubiquitously expressed pilus (Taktikos et al. 2015; Craig, Pique, and Tainer 2004; Craig, Forest, and Maier 2019). Without tfp, microcolony formation cannot occur and bacteria will remain planktonic. Microcolonies coalselence with neighboring microcolonies to eventually form larger aggregates [(Taktikos et al. 2015)](https://paperpile.com/c/s3IYV8/jJAy)

Force generated from tfp dynamics plays a critical role in *Ng* microcolony formation (Howie, Glogauer, and So 2005). In previous work, we modeled and observed experimentally cell movement in aggregates of *Ng.* We concluded that cells within aggregates, called microcolonies, confer differential motility depending on the location within the aggregate (Pönisch et al. 2018b). In Ng microcolonies have an outer perimeter layer of more motile cells and less motile cells in the core. As a result, we believe there is a connection between type IV pilus mechanical activity and bacterial physiology. To assess the relationship, we measured physiological responses of WT and retraction-deficient mutants, Δ*pilT*, in which pili are present and chemically unchanged, but do not retractile and thus exert no force (Dietrich et al. 2009) (Figure 1B). Both WT and retraction-deficient bacteria can form microcolonies, Δ*pilT* form loose microcolonies (Brown et al. 2010). We indeed demonstrate here that PilT functionality has a critical impact on physiological assays pinpointing key moments of the bacteriological life cycle: survival, antibiotic susceptibility responses and gene expression patterns. We surmise that the appreciation of the role of Tfp retraction forces in influencing bacterial physiology in Ng will both help fight an important human pathogen and continue to open the door on the exploration of physical forces in bacterial physiology.

### Results

#### Intercellular forces modify cellular survival of *Neisseria gonorrhoeae* cells.

Physical cues have been shown to affect the physiology and metabolism of many eukaryotic cells all the way to the ultimate choice between life and death. The forces exerted by the retraction of *Neisseria gonorrhoeae* type IV pili on human epithelial cells can trigger a force dependent cellular response in epithelial cells demonstrating the importance of mechanical cues in the life cycle of this human pathogen. The retractive forces powered mostly by the molecular motor pilT create a signaling hub underneath the bacterial cells recruiting actin and many other human proteins. Mutated bacterial cells deprived of the molecular motor pilT cannot exert similar forces and fail to recruit those human proteins. Yet, to the best of our knowledge, the global impact of the retractive forces of type IV pili on the physiology of *Neisseria gonorrhoeae* remains to be evaluated. We have shown previously that type IV pilus mediated forces within the microcolonies formed by Ng cells lead to a gradient of motility that can in turn lead to heterogeneous gene expression across the microcolonies. But we have yet to assess how these intercellular forces can affect globally the physiology of these bacterial cells. To tackle this problem, we have turned to the evaluation of the growth and survival of Ng bacterial cells in a static biofilm configuration under a stressors, antibiotic selection, and a force mimicry method, applying artificial vibration.

~~In a static biofilm configuration, a given number of cells is added to a fixed amount of liquid onto a static surface~~. We have added 5 x 107 cells to 1 ml of GCB medium per well on a 6-well plate. First, we compared the dynamics of growth and survival of WT cells versus cells deprived of the motor pilT, Δ*pilT*. Growth of both strains is comparable during the first 4 hours. Starting around the 8-hour time point the Δ*pilT* cells die prematurely compared to WT cells. ~~In one instance~~ there were approximately 9.5 times less surviving Δ*pilT* cells than WT cells at 24 h. After 48 hours all strains show no viable cells (Figure XX, or Data not shown). This is the first indication that a critical aspect of physiology, life and death, is controlled by the tfp-mediated intercellular forces in Ng.

Second, we tested if the premature death phenotype observed for Δ*pilT* could be rescued with the restoration of intercellular forces. A heterologous complementation of the pilT gene fully abolished the premature death phenotype. We then assessed whether mixing both WT and Δ*pilT* cells could also rescue the phenotype, the assumption being that the retractive forces generated by the WT strain would be sufficient to compensate for the retractive-deficient Δ*pilT*. We found that mixing the two strains can rescue the Δ*pilT* cells from premature death for at least 16 hours. Mixed colonies of cells ultimately die sooner than colonies of WT-only cells. This confirms our earlier demonstration that WT and Δ*pilT* cells segregate over time, thereby diminishing the amount of Tfp intercellular forces that the WT cells can exert or be subjected to. Nevertheless, this is a second indication that Tfp mediated forces can modulate survival in Ng.

We further tested the impact of mechanical forces on the survival of Ng cells by submitting cells to an external force by vibrating the plates in which the static biofilm assay was performed. We used three different protocols to apply vibration: First 3 hours followed by 21 hours of no vibrations (labeled: 3), first 3 hours without vibrations followed by 21 hours with vibrations (labeled: 21), and continuous vibrations for 24 hours (labeled: 24) (Figure 2C).

We observed that the rate of survival for WT was comparable, except at 21 H under VCN selection showed a 10-fold decrease in comparison to the control (no vibrations) (Figure 2D). We noted the rate of survival for Δ*pilT::*Kanwas not the same as no vibration control .In comparison to the control, Δ*pilT::*Kanincreased survival by 10-fold in both 3 and 24 conditions, while decreasing dramatically by 500-fold in the 21 condition. These results further demonstrate the importance of physical forces in regulating Ng physiology. Ng cells not only have the ability to exert forces through their pili but the ability to sense Tfp-mediated forces through a mechanism yet to be determined. Similarly to what has been since many times in the development of multicellular eukaryotic systems their seems to be specific time windows at which these mechanical cues are needed.

|  | *Bacterial cell type* | |
| --- | --- | --- |
| *Vibration duration* | *Wild-type* | *ΔpilT* |
| 3H | 100-fold ­↑ | < 10-fold ↑­ |
| 21H | < 10-fold ­↑ | Same as WT control; KAN resistance: 10-fold ↓ |
| 24H | 10-fold ­↑ | < 10-fold ↑­ |

Table X: Vibration survival summary in comparison to no vibration. Vibration force affects survival differently than no vibration.

#### Acceleration of microcolony assembly decreases antibiotic susceptibility

In order to assess whether retractive force plays a direct physiological role in Neisseria during microcolony formation, we carried out a series of assays measuring microcolony susceptibility to a battery of antibiotics as a function of (interaction) time to colony formation. If pilus retraction during bacterial microcolony formation is important for normal microcolony development, then sensitivity to antibiotics—previously demonstrated to be a function of pilus retraction (REF)—can be used as a proxy to measure the physiological importance of pili retractive forces. Two Neisseria strains are compared: wild-type (WT) and *ΔpilT* (a retractive force-deficient mutant. Briefly, planktonic cells are either spun down in a centrifuge or left to settle and form microcolonies based on the ability to produce retractive force (Figure 3A). For the WT strain, the brief spin accelerates microcolony formation by immediately increasing the local concentration of bacteria on the culture plate surface. For the force-deficient mutant, the opposite is true: the spin inhibits microcolony formation by sticking the bacteria directly to the culture plate and, because they are non-motile, do not allow the bacteria to form the loose aggregates that would have formed had they been allowed to settle by gravity. Prior to the start of each assay, cells were either spun down or not, and then incubated at 37C for 2H to allow for microcolony formation. Following this incubation, spun and unspun cells were incubated for 1-hour in the presence of either growth media without, or supplemented with one of six antibiotics: Erythromycin (Erm; 50µg/mL), Chloramphenicol (Chl; 250µg/mL), Nalidixic acid (NA; 30µg/mL), Kanamycin (Kan; 80 µg/mL), Ciprofloxacin (Ci; 0.12 µg/mL) and Cephalexin (Ce; 50µg/mL); the former three being bacteriostatic and the latter three bactericidal at their respective minimum inhibitory concentrations (MIC). Following the 1-hour incubation in the presence or absence of antibiotics bacteria microcolonies were broken apart and assessed for viability.

For WT bacteria, there was a significant reduction in cell survival of the bacteria lacking the spin down step prior to treatment exposure for two of the three bactericidal antibiotics (Kan and Cip) and a reduced cell survival for all other tested antibiotics when compared to their respective spun down controls (Figure 3B). For the force deficient mutant, there was little to no difference in cell survival for either the controls (no antibiotics) or four of the six tested antibiotics (Ceph, NA, Erm, Chl). Another of the bactericidal antibiotics, Cip, killed all force-deficient bacteria, regardless of whether they were spun down prior to exposure or not.

These findings suggest that tfp generated mechanical forces do indeed play an important role in modulating bacterial antibiotic susceptibility: even though the microcolonies have the same size whether spun or not, their susceptibility to the antibiotics can be orders of magnitude apart. The most likely candidate is the difference of tfp retraction forces experienced by the bacteria (Persat, Inclan, et al. 2015b; Persat 2017).

Our antibiotic-exposure survival assay demonstrates for the WT strain, spinning down the bacteria, or accelerating microcolony formation dynamics, offers a protective effect for the bacteria. The protective effect is present against all tested antibiotics, despite each having differing modes of action, would indicate a global protective mechanism. Accelerating microcolony formation reduces the amount of time each single planktonic bacteria cell is exposed to each of the antibiotics. Pili-pili interactions of microcolonies leaves less free pili for interaction with the antibiotics. In contrast, for the retractive-force deficient strain, time spent as free-floating single cells (either in loose aggregates when allowed to settle by gravity or when stuck to a cell culture plate) does not increase cell death (Figure 3B) under five of the six antibiotics tested..

#### pilE and pilT gene expression have differential spatiotemporal patterns

From previous work in our lab, we have modeled the motility of cells in microcolonies formed on a motility gradient [(Pönisch et al. 2018)](https://paperpile.com/c/s3IYV8/Dakm). A motility gradient is formed because increased pilus-pilus interactions lead to less movement; therefore cell motility is stronger at the perimeter of the microcolony. We aimed to measure whether motility was associated with gene expression. To measure the spatiotemporal transcriptional activity of developing *Ng* microcolonies, we used pilus-related promoters that drive an mCherry reporter cassette in live cells. mCherry emission fluorescence thus was a proxy for transcriptional activity. mCherry intensity of individual microcolonies was captured by epifluorescence imaging at 3- and 7-hours. Time points were chosen since 3-hour microcolonies achieve stable size (~20µm in diameter), and at 7-hours. For measurement consistency we focused on microcolonies with spherical shape. To get a consistent image plane, it is sufficient to image the mid-section of each microcolony.

We measured spatiotemporal intensities in developing *Ng* microcolonies (Figure 4A). Pilus-related promoters demonstrate variable activity across the microcolony spatial plane and time. For the quantification of the spatial gene expression profiles of colonies, we define three features: homogeneity , directionality and roughness . The homogeneity is a measure of the evenness of the fluorescence intensity profile. For , the profile is completely homogeneous, for , it is heterogeneous and the smaller it is, the more heterogeneous. The directionality quantifies if regions of higher intensities values, and thus stronger gene expression, are located in the core or at the perimeter of a colony. The roughness R quantifies the standard deviation of the gradient of the fluorescence signal. It measures how smooth or rough the fluorescence signal is and is a measure of if intensity changes happen on small or long length scales. In Fig. 4B-D, we present the qualitative behavior of these features, in the Methods section we provide quantitative descriptions.

First, we investigate the gene expression profiles of colonies formed by mCherry WT cells (Fig. 4A and 4B) . There is no significant difference in directionality and homogeneity between the colonies, suggesting no change in gene expression patterns (Fig. 4C and 4E). The 7-hour colonies express a slightly weaker roughness than the 3-hour colonies (Fig. 4E), potentially due to the lack of few brighter cells that we observe at 3 hours. As expected, the mCherry does not alter the gene expression profile within the colony. We next examined colonies formed by *pilE*-mCherry cells. In this case, we find that at 3 hours, cells are significantly more homogeneous than at 7 hours (Figure 4C). The directionality is unaltered and confirms that the majority of fluorescence signal is located in the core of the colony (Figure 4D). At 7 hours the profile has a lower roughness , again mediated by the lack of extremely bright cells within or at the edge of the colonies (Figure 4E). Even though such bright cells are lacking, the profile is still less homogeneous at 7 hours and we conclude that gene expression of pilE is time and spatially dependent. We also studied the gene expression of *pilT-mCherry* at 3 hours and 7 hours. We find that neither the homogeneity, nor the directionality or roughness change significantly. Gene expression of pilT seems to be not changing in this time interval not dependent on time.

#### External forces disrupts gene expression patterns

We hypothesize that spatiotemporal patterning of the pilus genes could also be regulated by a mechanosensitive pathway. Therefore, we assessed the effect of external forces on transcriptional activity of promoter reporters. External force by vibration (~60Hz) was applied to live developing microcolonies for 7 hours. After incubation, microcolonies were imaged with fluorescence microscopy, and the integrated intensities of vibrated and not vibrated microcolonies were compared. In Fig. 5D-F, we show the spatial change of the gene expression profile of colonies formed by mCherry cells. While the homogeneity is increasing when the colonies are vibrated, we find that the difference is only weakly significant. Independently of the vibrations, the directionality is positive, corresponding to a brighter core within the colonies and the roughness is not significantly different. We conclude that vibrations have only a weak impact on the gene expression patterns for *pilT*-mCherry colonies. For *pilE*-mCherry, we find a more clear impact of vibrations (see Fig. 5B-D). Vibrations significantly increase the homogeneity , while leaving the directionality and roughness unchanged. When a *pilE*-mCherry is vibrated, the spatial gradient of gene expression is reduced and becomes more homogeneous. Finally, colonies formed by *pilT*-mCherry cells keep their level of homogeneity (see Fig. 5C), and independent of vibrations keep a positive directionality (see Fig. 5D), corresponding to the majority of gene expression taking place in the core of the colony. Additionally, the roughness , is only weakly affected by vibrations (see Fig. 5E). We conclude that *pilT*-mCherry colonies are not affected by vibrations. The results of this experiment suggest that pilE promoter and consensus sequence, lacUV5, are sensitive to vibration, and/or continuous vibrations induce a global stress response. pilE promoter is regulated by a diverse set of transcription factors, which will lead our future direction of this study ((Hill, S. A, 1997; Masters T. L, 2016).

### Discussion

The combined role of chemistry and physics in organising the biology of multicellular organisms, already put forth by Alan Turing in his seminal paper[(Howard, Grill, and Bois 2011)](https://www.zotero.org/google-docs/?Yetu3k), is now widely accepted. Yet, seeing bacterial biofilms as multicellular organisms and studying the impact of physical forces on their physiology is still in its infancy[(O’Toole, Kaplan, and Kolter 2000; Stoodley et al. 2002)](https://www.zotero.org/google-docs/?74s6KD). Nevertheless, mechanomicrobiology, the study of the role of physical forces in the prokaryotic world is emerging as an important and active area of research[(Dufrêne and Persat 2020; Wong et al. 2021)](https://www.zotero.org/google-docs/?TvR52q) with the potential to understand the depth of bacterial physiology and design new tools to combat disease . The dynamical potential of bacterial appendages like Type IV pili to read mechanical cues and affect bacterial physiology at the single cell level has been demonstrated in species like *Caulobacter crescentus*[(Ellison et al. 2017)](https://www.zotero.org/google-docs/?nc3zoI) or *Pseudomonas aeruginosa*[(Persat et al. 2015)](https://www.zotero.org/google-docs/?t2hKfX). It is widely accepted in the community that bacteria rarely are by themselves . While highly organized bacterial adhered communities called biofilms have been described as the most common bacterial lifestyle for decades, it seems that the structure associated with the early stages of biofilm formation, the microcolony, might be ubiquitous[(Bjarnsholt et al. 2013; Mark Welch et al. 2016)](https://www.zotero.org/google-docs/?Y8n7Nh). In the case of the etiological agent of gonorrhea, *Neisseria gonorrhoeae*, the microcolony has long been regarded as the main infection unit.

In this study, we show that the Ng microcolony whose formation and maintenance mostly depends on Tfp has also its physiology controlled by the mechanical interactions between the cells that form the microcolony. The first line of evidence is the fact that microcolonies formed by ΔpilT bacteria, and are thus retraction-deficient, die more quickly than their WT counterparts in a static biofilm assay. We can partially complement this phenomenon by mixing WT and retraction-deficient cells. The segregation of WT and ΔpilT cells within microcolonies that we have documented before indicates that not all ΔpilT cells will be experiencing WT Tfp retraction forces giving a reason for not fully restoring the WT phenotype. We have also shown that ΔpilT microcolonies submitted to external forces by vibrating the microcolonies can restore the same level of survival as the WT cells. Interestingly the timing of the application of the external forces is critical in being able to restore WT survival. This is reminiscent of the existence of time windows for the efficacy of certain cues during development. Tfp both power the dynamical formation of microcolonies and are the most obvious choice for being the mechanosensor that triggers gene expression changes. Ng does not seem to possess genetically the two pathways that have been explored in Tfp based mechanosensing in single cells (the ChP chemosensory pathway and c-diGMP pathways). In the case of Ng, the time for impact of forces on gene expression seems to be on the order of hours compared to minutes in other species. It is tantalizing to think that Tfp mechanosensation could be done differently in Ng, maybe directly linked to a change of quaternary structure of the pilus itself[(Biais et al. 2010)](https://www.zotero.org/google-docs/?zWIRKx).

The mechanisms of resistance to antibiotics have been mostly characterized in planktonic bacteria, yet, even though microcolonies and biofilms are the most prevalent. An obvious issue within biofilms is the ability of the antibiotics to penetrate within the biofilm[(Lebeaux, Ghigo, and Beloin 2014)](https://www.zotero.org/google-docs/?zwvCt5). This is not an issue in the case of a Ng microcolony with a diameter of less than 20 microns. Biofilms also represent a different physiological state for the bacteria, and the link between metabolism and resistance to antibiotics is now coming to the fore[(Stokes et al. 2019; Baquero and Levin 2021)](https://www.zotero.org/google-docs/?hVf9Vr). In many systems reducing bacterial metabolism is a powerful and often underestimated means to gain resistance to a wide range of antibiotics[(Lopatkin et al. 2021)](https://www.zotero.org/google-docs/?3q2EK0). So if mechanical cues can modify bacterial physiology they can in turn modulate bacterial susceptibility to antibiotics. A recent example of feedback loop between mechanical cues and resistance to antibiotics was published in the case of single *Caulobacter crescentus* cells[(Banerjee et al. 2021)](https://www.zotero.org/google-docs/?3f6G8u). *Vibrio cholerae* microcolonies are also physically modified during antibiotic treatment[(Díaz-Pascual et al. 2019)](https://www.zotero.org/google-docs/?XYZxVT). Recently it was shown that antibiotic treatment could change the interactions between cells within a Ng microcolony[(Cronenberg et al. 2021)](https://www.zotero.org/google-docs/?RkbpNy). In our study, we show that by affecting the timing of formation of Ng microcolonies, and thus the mechanical history of the cells within the microcolonies, we can change the sensitivity to multiple antibiotics by orders of magnitude. This exemplifies the importance of the feedback between mechanics and physiology. Affecting the mechanical state of a microcolony could have a dramatic impact on its susceptibility to antibiotics and opens the door to reducing antibiotics resistance.

In this study, we show that both internal and external forces can affect the spatiotemporal expression of genes within Ng microcolonies. This provides further evidence that the mechanical forces within microcolonies can shape their physiology. Our results converge toward the proposed model: as the cycles of elongation and retraction of Tfp bring Ng cells together to form microcolonies the same Tfp are sensing the forces that the cells are experiencing. These mechanical cues are integrated over hours to lead to the expression of specific genes in defined spatiotemporal patterns. The understanding of this entire mechanism, while beyond the scope of this study, is ripe with possibilities, from understanding antibiotic resistance mechanisms to optimizing bacterial growth. The primary questions would be to identify the mechanosensor and the signal transduction relay mechanisms. We will get to work now:-)

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Mechanosensitive signalling mechanisms in biological systems demonstrate their important roles in both animal and bacteria cells. Bacteria survive in dynamic environments where they move, adhere to surfaces and form multicellular units using extracellular structures. Consequently, the use of extracellular appendages to engage in motility or form multicellular structures, cells are exposed to mechanical forces. Similarly to muscle cells, we expect that mechanical forces play a key role in bacterial physiology. We explored the global response to type IV pilus retraction forces on physiological contributions to the microcolonies, a bacterial multicellular structure, with a combination of traditional survival assays and *de novo* experimental methods.

Summarize what we did:

* survival of assays of wild-type and retraction-deficient bacteria. We found that retraction-deficient bacteria survive 9.5 times less than wild-type cells. Without a functional copy of the pilT protein, viability is compromised. In a rescue experiment where retraction-deficient bacteria were mixed in equal concentrations, the presence of wild-type cells with retraction-deficient bacteria was sufficient to rescue the survival rate.
* antibiotic exposure and survival assays.
* gene expression and vibration assays: Gene expression in individual microcolonies demonstrate spatiotemporal heterogeneity. Collectively, the results of the fluorescence reporters assays and the vibration assay both suggest an association between differential forces in microcolony and differential spatial gene expression. The force transduction mechanism as a transcriptional signal is currently unknown, however, we propose the pilus machinery as a strong candidate as a transcriptional regulator of physical forces.

Describe results in alignment with other literature.

* Microcolonies modulate physiological activity, like survival after antibiotic treatment [(Cronenberg et al. 2021)](https://paperpile.com/c/s3IYV8/92xM)
* <https://pubmed.ncbi.nlm.nih.gov/28068612/> This review highlights the link between force and biochemistry.
* <https://www.nature.com/articles/s41579-019-0314-2.pdf> A review on mechanomicrobiology, even though it is not the focus, there are some things about the connection between gene expression and mechanics.
* <https://www.nature.com/articles/s41567-020-01079-x> Links cell growth and morphology to antibiotics [(Banerjee et al. 2021)](https://paperpile.com/c/s3IYV8/xw4M)

Propose new ideas based on our results:

* Highlight that microcolonies are the common structure as opposed to the most popular microbial mats.
* Advocate that the type IV pilus is the global regulator
* Perhaps creating variation between microcolonies produces physiological variation necessary for establishing viable aggregates. Sorting cells? Essentially, we know microcolonies as a population have heterogeneous properties in viability, gene expression, and morphology. We predict that differential physical induction of heterogeneity is a mechanism for producing diversity. In future work, it would be insightful to measure how genetically identical cells respond to various mechanical input, and vice versa, genetically different cells respond to consistent mechanical input. Perhaps some cell types will react and others not, and this leads to some synergy.
* Other ideas one can try and maybe in some form mention in the discussion (sorry, a bit long. I was just brainstorming):
  + 1. What about a movie to see how gene expression patterns emerge and how this is linked to cell motility + cell interactions. We would need a fancy microscope of the Maier group though. There, one could also think about some theory and combine this with our models.
  + 2. Magnetic tweezer to pull on an individual colony and see how it reacts to antibiotics. This would be connected to the heterogeneity stuff above. (Magnetic since I would hope that like this we don't burn the cells). And another thing: What happens if we then let this colony interact with a colony that was not shaken?
  + 3. Mixture of different cell types that form biofilms together and see how they alone and together behave in the same experiments.
  + 4. See how antibiotic resistance is affected when cells grow not on a petri dish but on real cells.
  + 5. In the Banerjee manuscript, they show that antibiotics change cell shape. Is that also the case for N. gonorrohoeae. If yes, how does this affect the colonies? And connected to that, is cell shape changing if we add mechanical forces to them? Connected to that: Nicolas. Have you ever estimated with what forces you need to pull a diplococcus so that its shape changes significantly? Is it much higher than 1 nN? Would be interesting to estimate.
  + 6. This was something Nicolas or Kelly mentioned before. Would one see the same behaviour (antibiotics & survival) if, instead of having cells in a colony, have them on a substrate that is periodically stretched and compressed and thus creating mechanical force on the cells?
  + 7. Other genes for the gradients.

## 

## Methods

#### Culture conditions

*Neisseria gonorrhoeae* was cultured on Gonococcal Broth (GCB) with VCN (Nystatin, Colistin, and Vancomyocin) agar plates with Kellogg’s supplements (Dextrose, L-Glutamine, Cocarboxylase, ferric nitrate) (Schoolnik et al. 1984). Incubated at 37°C and 5% CO2. In order to have the most live cells, it is advised that the plate be incubated no longer than 18 hours. Frozen bacterial stocks are stored in freezing media (20% glycerol with 80% GCB) stored at -80°C.

#### Promoter reporter construction

Gibson Assembly method (NEB #E2611S) ligated DNA fragments for 60 minutes at 50°C. Genetic cassettes of promoter, mCherry and antibiotic resistance genes were inserted into locus CP003909 at 1489951bp (Dillard 2011). Primer design using Geneious 8.1.6 software. Verify ligation by TAE ethidium bromide gel electrophoresis (RE Biotechnology Grade, Amresco). Ligate desired DNA fragments for 60 minutes at 50°C. Amplify ligated construct using GoTaq polymerase master mix (Promega #M7122) along with 10µM forward and reverse primer. To transform the construct in *Ng*, spot 10µl of verified ligated construct (~100 ng/µl) onto GCB agar plate, and inoculate with Neisseria gonorrhoeae WT bacteria. Incubate overnight at 37°C 5% CO2, then select for positive colonies with construct. Sequenced all constructs using GeneWiz.

Promoters regions were identified using SoftBerry Bacterial Promoter Prediction Program (BPROM), which predicts regulatory elements associated with promoter regions (Solovyev 2011). BPROM predicts regulatory elements like transcriptional start sites, transcriptional binding sites, -10 box and -35 box. A genetic cassette composed of promoter fused to mCherry gene and antibiotic selection gene, which was chromosomally inserted to the same loci for all constructs (Supplemental figure). As a non-pilus expressing control construct, we used an *E. coli* LacUV5 consensus sequence promoter to drive mCherry expression.

#### Primers

| Promoter | Forward Primer | Reverse Primer | Promoter length (bp) |
| --- | --- | --- | --- |
| pilE | gggtatagagcagaacggatCCCCACCCAACCCACCCG | cctcgcccttgctcaccatCATCCGTTCTGCTCTATACC | 393 |
| pilT | gggtatagagcagaacggatTCCGCCAATTCCTCCGTTTTG | cctcgcccttgctcaccatCATCCGTTCTGCTCTATACCC | 368 |
| pilU | gggtatagagcagaacggatCACACAACCGCC | cctcgcccttgctcaccatCGTTAGCTTCTTTTCGG | 163 |

*LacUV5 consensus sequence*:

Cgctgctgccgctgtaatacggcttgacactttatgcttccggctcgtataatgtgtggatagtgggaggaaagc

#### Microcolony assays

Filter GCB with 0.2 µm syringe filter (VWR #28145­477). Following 14-16 hours of growth, remove cells with polyester swabs (VWR #22222-046) and resuspend in 1 mL GCB plus supplement. Vigorously shake bacterial suspensions for 2 minutes using ‘Disruptor Genie’. Vortexing ensures single non-aggregated cells. Dilute cultures to OD600 0.7 in GCB. Add 100μl of dilution to 900μl GCB supplement in 6-well plate (Costar #3516) and incubate at 37°C at 5% CO2. 500μl of culture was transferred to Attofluor chambers (Life Technologies #A7816).

**Imaging**

All images were obtained on a Nikon Ti Eclipse inverted microscope equipped for epifluorescence and DIC microscopy all under an environmental chamber maintaining temperature, humidity and CO2 concentration. The objective used is an oil immersion Plan Apo VC 100X objective, N.A 1.4, refraction index 1.51. DIC images were captured at 100ms exposure, gain 2.3X, binning 1x1. TexasRed images were excited at 555nm, captured at 500ms exposure, gain 1.5X, binning 1x1, and laser power of 50%. The camera used was digital SLR CMOS Nikon DS-Qi2, 16.25 megapixel.

#### Image analysis

*General information:* For each colony images of two channels were used: DIC channel allowed for the identification of the colony boundaries, the fluorescent channel allowed for the analysis of the gene expression profile. R2019a was used for the analysis of fluorescent patterns within colonies.

*Edge detection: Since robust automatic segmentation of DIC images remains a challenging task up to this day, we manually segmented the cross section of colonies from the DIC channel using Fiji (Schindelin 2012, doi: 10.1038/nmeth.2019), see Fig. S1A and S1B.* We manually disregarded colonies that formed due to a recent coalescence event and that typically have a dumbbell shape or a clear signature of the two previous colonies in the fluorescence channel. We only picked colonies with a not too rough surface by only considering colonies with a solidity > 0.75 (ratio of the area and the convex area of the binary shape of the colony). Additionally, we only picked colonies that were spherical by only selecting colonies with an axis ratio > 0.75 (ratio of short and long axis of an ellipse fitted to the binary colony shape). Additionally, we computed the radius of the circle with the same area as the binary shape of the colony and only pick those colonies with radii above 5 µm and below 10 µm.

*Background subtraction:* To estimate the background of the fluorescent channel, we manually choose a rectangle in the fluorescence images without any cell or colonies and calculate the mean intensity in this rectangle. This mean intensity is subtracted from the fluorescence image.

*Estimating features of the fluorescent patterns within colonies:* We define three different features to quantify different characteristics of the fluorescence patterns within microcolonies.

The first quantity is called homogeneity and is the ratio between the area of the colony that includes the brightest pixels that account for 50% of the summed fluorescence of the colony and the total area of the microcolony (see Fig. S1C). It is given by the equation:

and is a measure of how homogeneous the pattern within a colony is. For , the profile is completely homogeneous, for , the majority of the fluorescence signal, and thus the majority of the molecular marker, is accumulated in a small region of the colony.

Since the homogeneity does not quantify at what location the fluorescence signal is accumulated, we define the directionality . Therefore, we perform a distance transform of the binary image of the colony (see Fig. S1D). The directionality results from the mean distance from the colony surface of the brightest pixel that account for 50% of the overall fluorescence and the pixel that account for the other 50%, , and is given by

It is positive if the majority of bright spots are within the colony core and negative if the majority of bright spots are near the colony perimeter. This quantity cannot exceed an absolute value of 1.

The last quantity, called roughness , is a measure of how smooth or rough the fluorescence pattern of the colony is, e.g. if it is characterised by a single large bright core or multiple bright spots. To this aim, we first apply a two-dimensional gaussian filter to the fluorescence kernel with a standard deviation of the smoothing kernel of 0.5 µm. Then, we estimated the gradient magnitude using the *Matlab* function imgradient() (see Fig. S1E) and, computed the mean and standard deviation of the gradient magnitudes of the colony and finally defined the roughness as the coefficient of variation:

This value is larger the rougher the profile is.

Illustrations of the qualitative behavior of the three quantities can be found in Fig. 5C-E. Microscopy

#### Survival and antibiotic resistance assays

Bacteria were streaked from frozen stocks and incubated overnight at 37°C, 5% CO2 and humidity. At 24hr, colonies were lawned and incubated for 18hr at 37°C, 5% CO2 and humidity. 18hr lawns were resuspended in 1 mL GCB, shaken 3 min on a disruptor, and assayed for OD600nm. Bacteria were then normalized to an OD600 0.7 and 100µl used to inoculate 1 mL of GCB (~5x107 cells). Plates were either spun down at 3500rpm, or not, and bacteria were then incubated at 37°C, 5% CO2 with humidity for 2-hour before addition of the selective agent. 1 mL of 2X concentration antibiotics were added following a pre-incubation period and left for either 1 hour. Following incubation under antibiotic selection, cells were scraped, disrupted for 3 min, diluted in a 1:10 dilution series, plated on agar and grown overnight at 37°C, 5% CO2 and humidity. Control for WT, ∆*pilT::Kan,* ∆*pilT-*complement, and WT, ∆*pilT::Kan* mixture in growth and 48-hour survival assay.

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