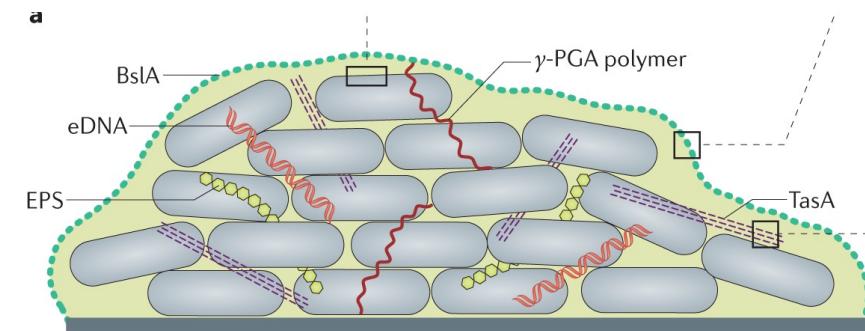
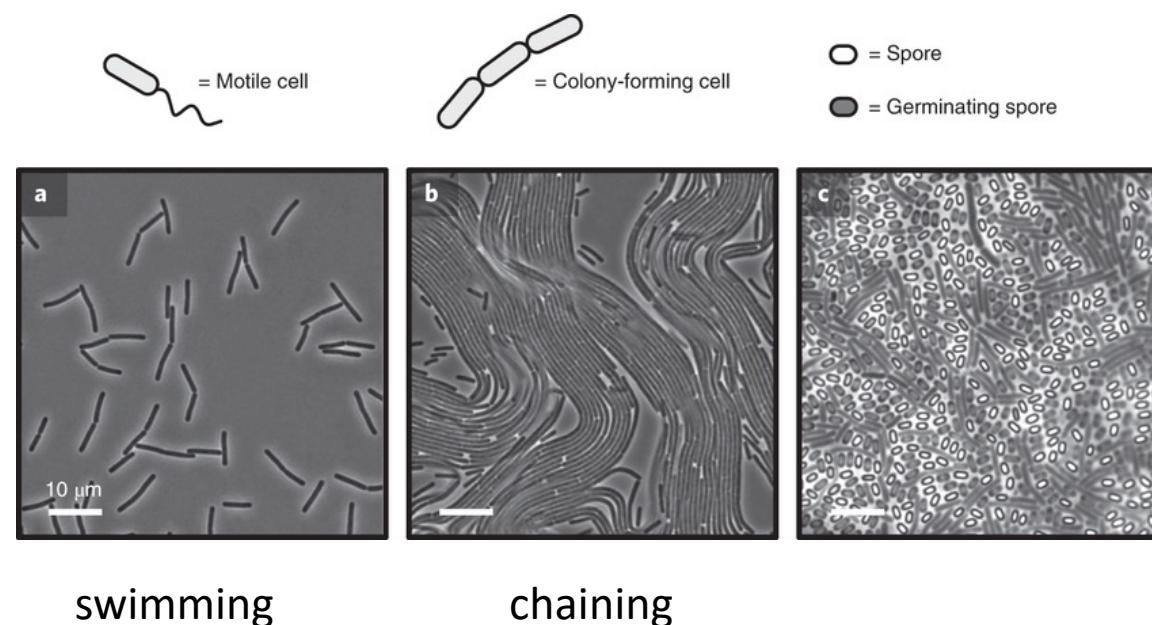
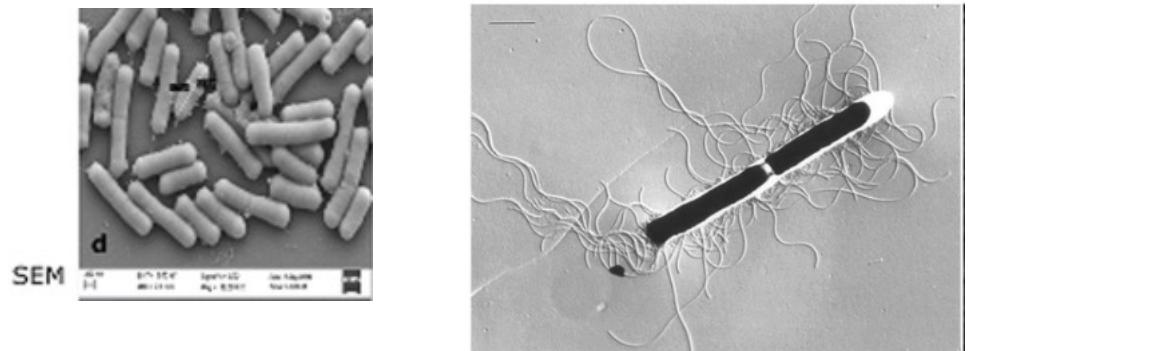


30NOV

# Regulation of Heterogeneity in *B. subtilis* Biofilm

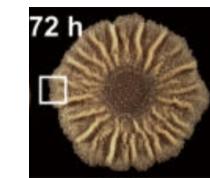
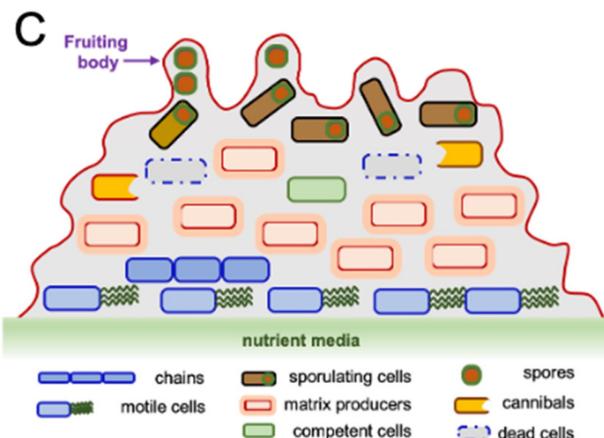
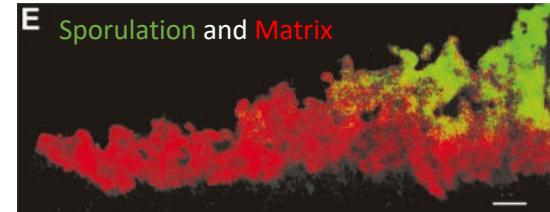


Wrinkles  
Channels

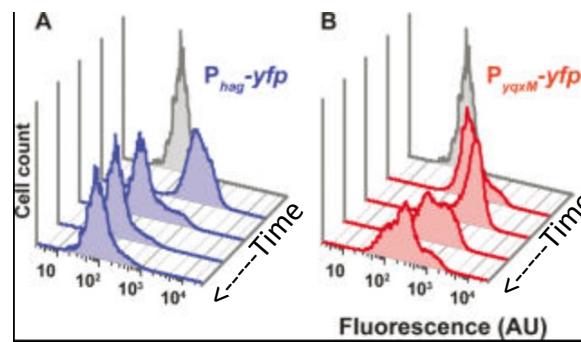
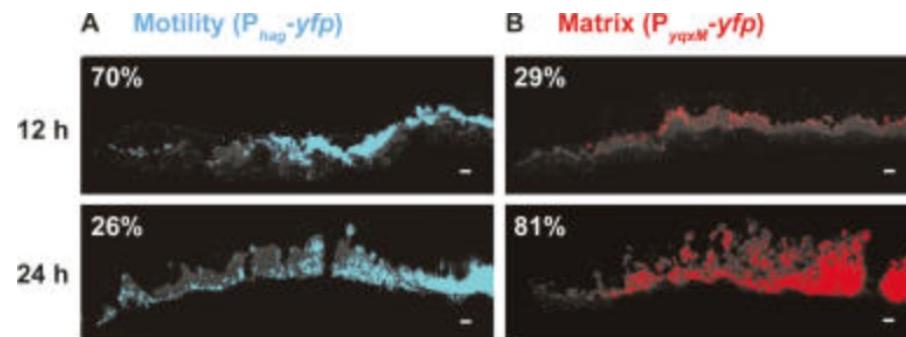
# Observations of Biofilms

Transcription of the genes needed for **motility** is **inversely correlated** with that of the genes needed for biofilm **matrix** production.

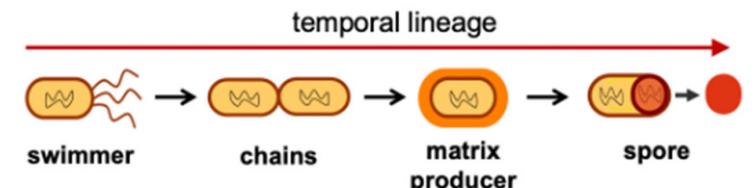
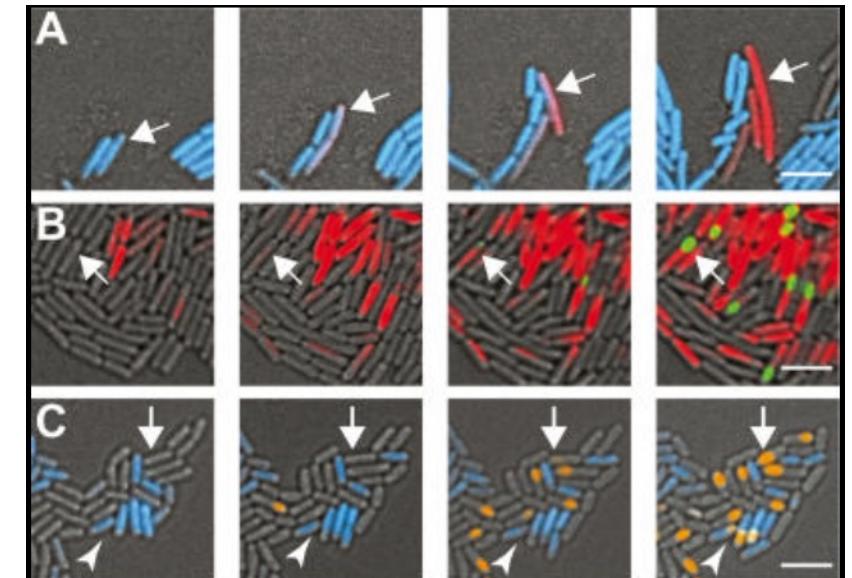
## Spatial heterogeneity



Grey – bg bacteria  
Blue – motile cell (*Phag* :: CFP)  
Red – matrix producing cell (*PtagA* :: RFP)  
Others – sporulating cell (*PsspB*::others)



## Temporal heterogeneity ⏲



# Time evolution of colony's gene expression

Lab's observation

Early: The subpopulation at the edge become *tapA* expressor

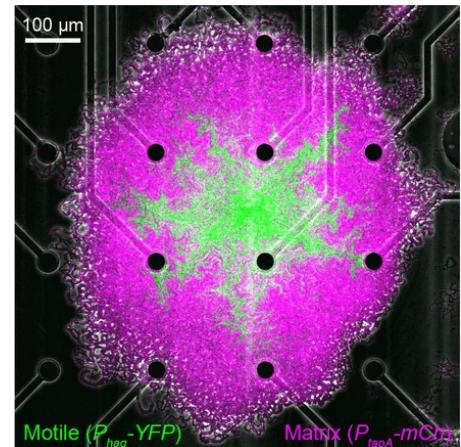
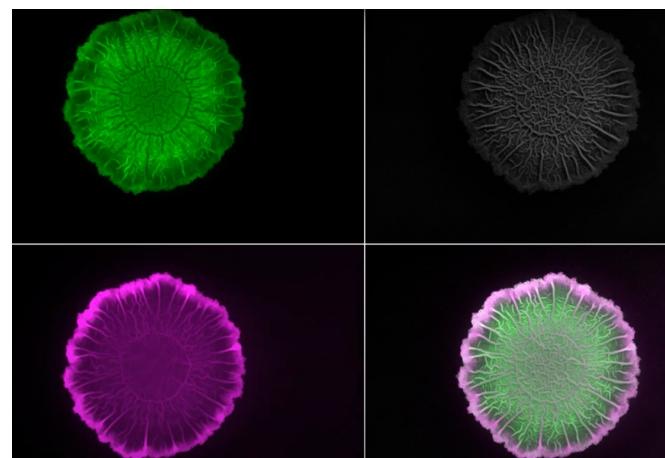
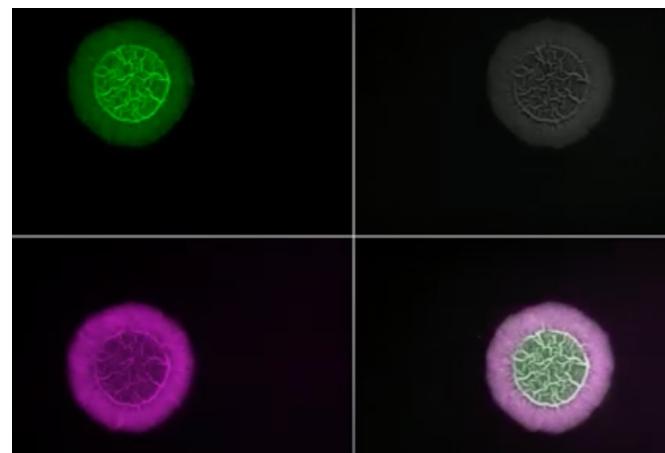
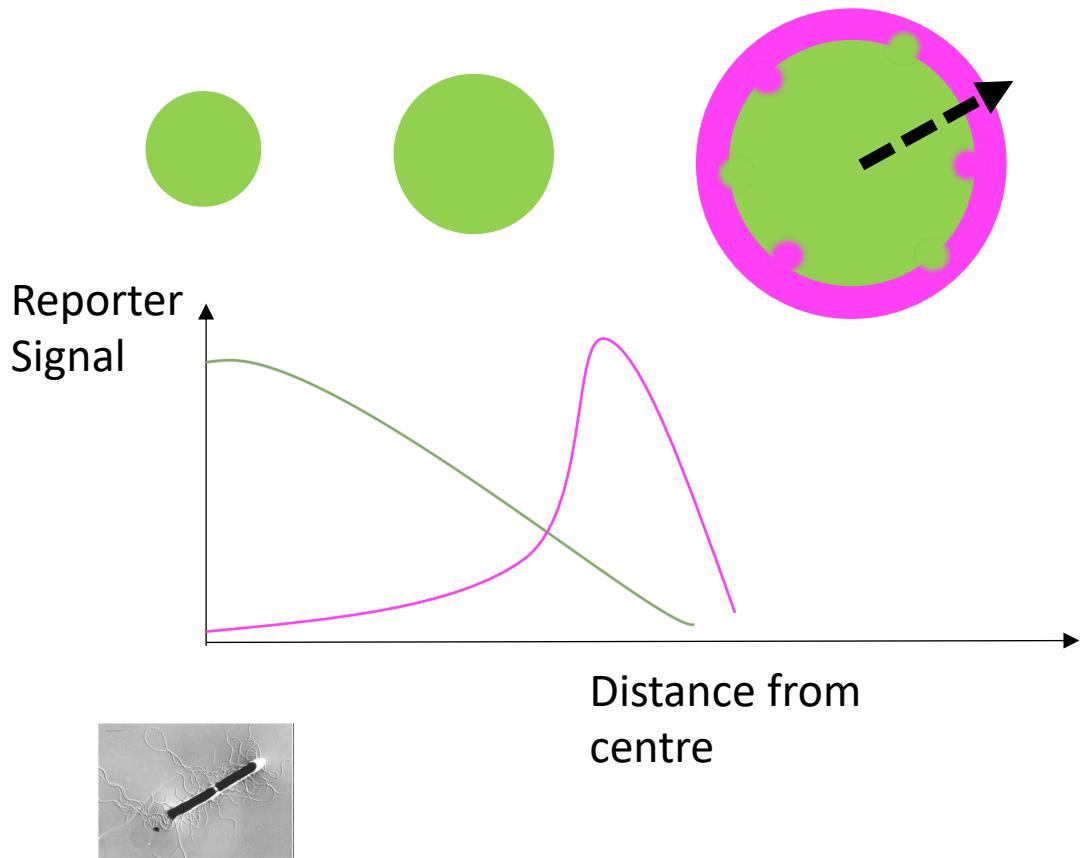
## Support

"First, upon initial cell loading, colonies did not exhibit distinguishable patterns of motile or matrix cells. Second, after approximately 16 hours of growth, biofilms had organized into a distinctive pattern, with a fractal-like population of motile cells surrounded by matrix-producing cells "

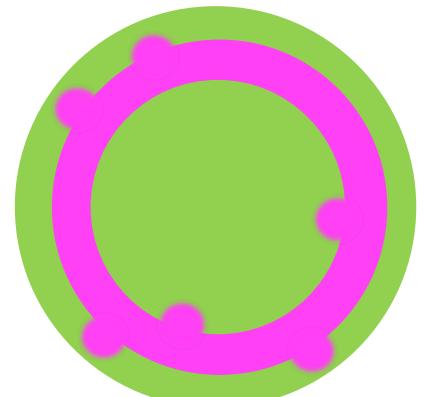
<https://www.biorxiv.org/content/10.1101/2022.05.10.491419v1.full>

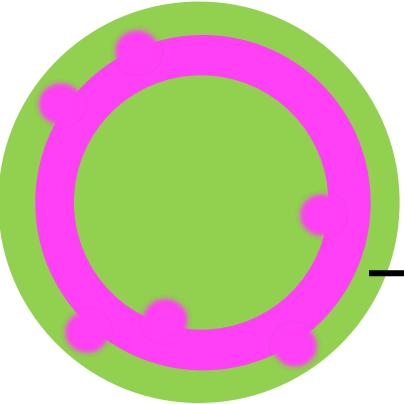
Green – motility cell (*Phag*)

Pink – matrix producing cell (*PtapA*)



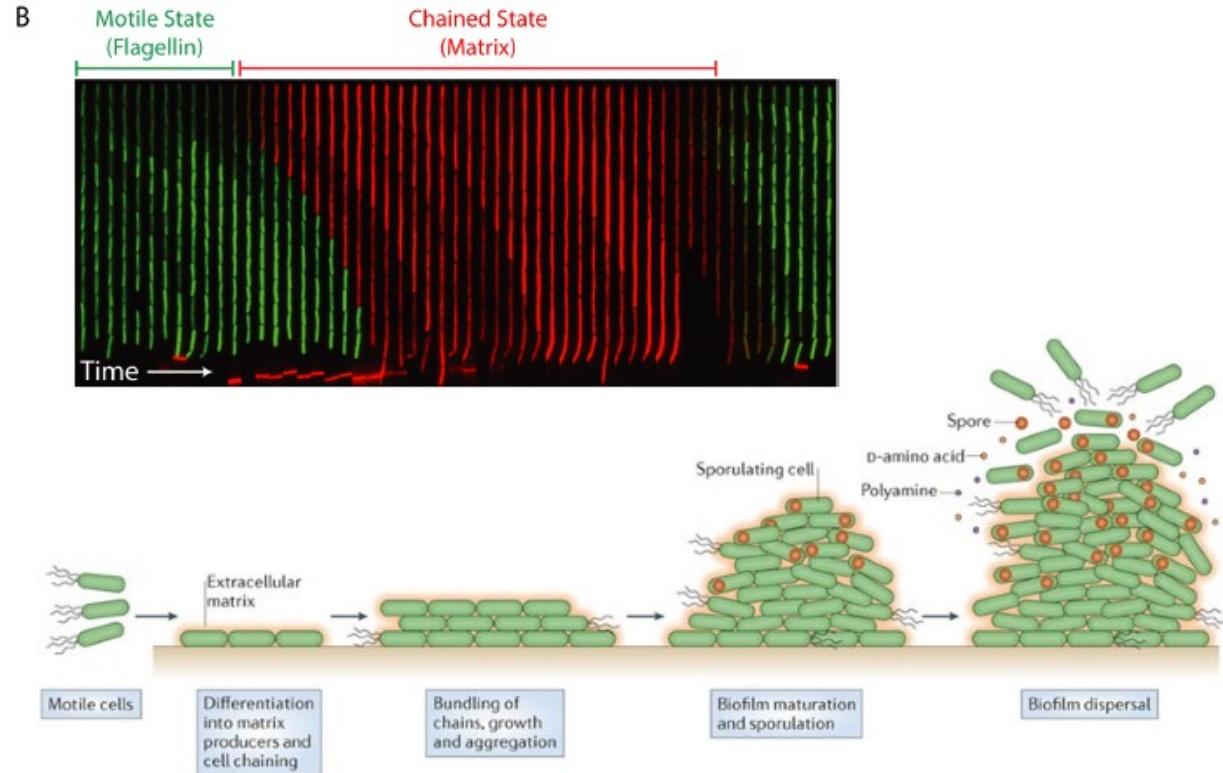
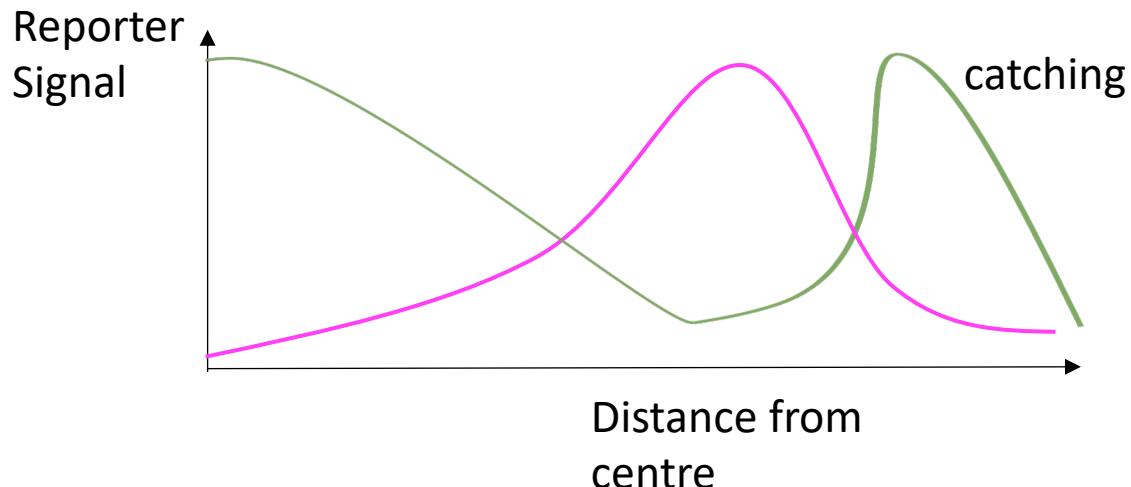
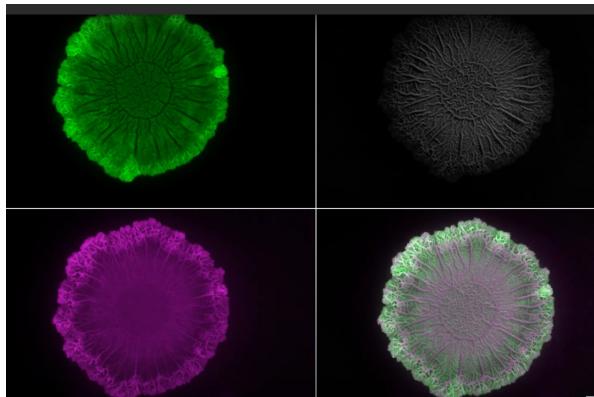
But then... 🚀





## Observation of potential switching

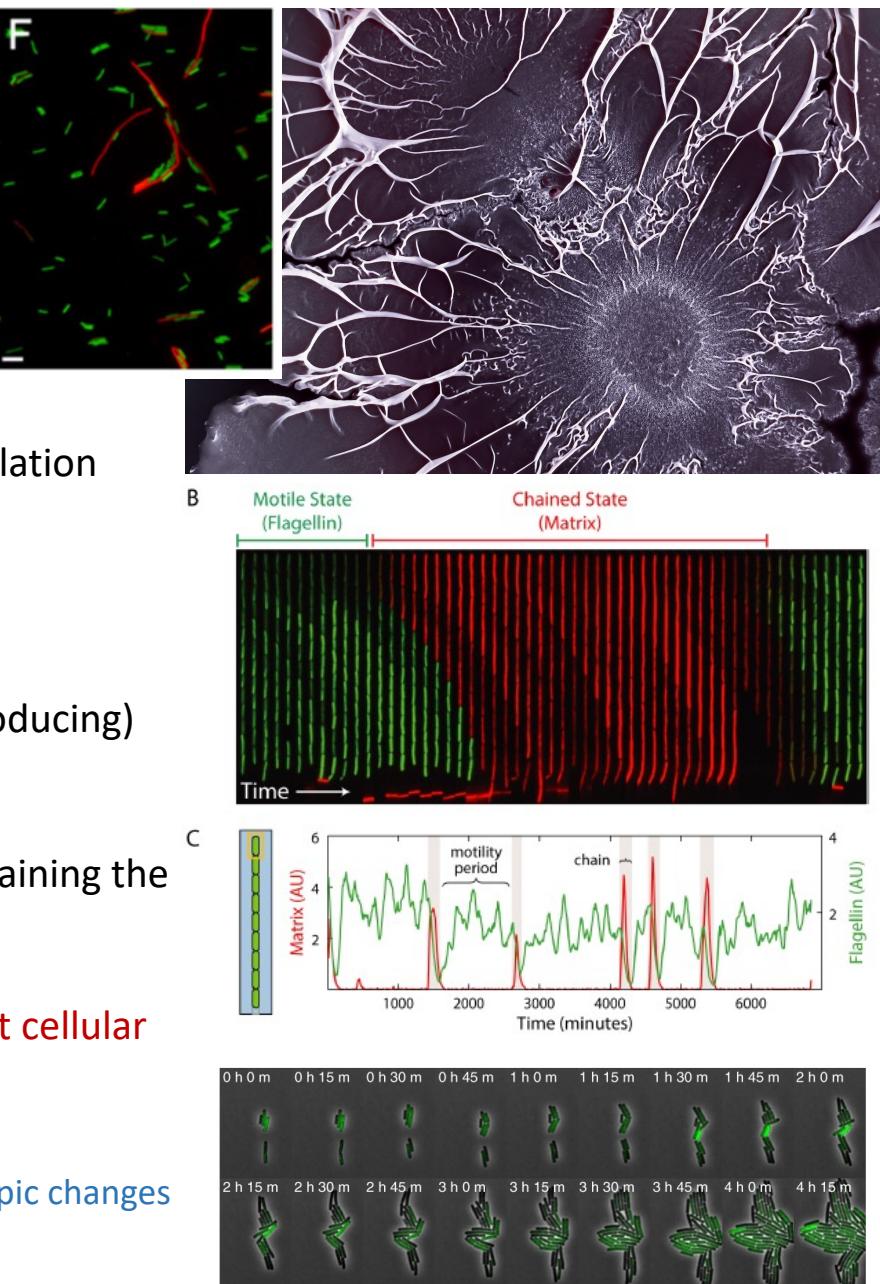
- A long time: the second layer of *hag* expressors emerge, which may be
  - H1: the *tapA* expressor at the edge that **switch** back to *hag* expressor
    - Likely – but still no confirmed observation in the biofilm 🎯
  - H2: the *hag* expressors previously in the middle **escape** to the edge
    - ✅ Nikhil and Joe's work: Confirm that the escape can occur (and that it needs wrinkle in the biofilm... another story)
  - H3: growth rate of peripheral motile cell > growth rate of matrix cell



# Summary: Why study regulation?

Many bacteria species stays in matrix (that they secrete) creating a so-called “Biofilm”

- Together evolve with time
  - It is, collectively, spreading in 2D and forming 3D structure
    - **But how? –what/how important different physical forces**
    - **And Why? -what are advantages of wrinkles**
- Has a division of labor
  - Each cell has a role – motile, matrix-producing and spore-producing sessile subpopulation
  - They exist in specific spatial pattern (both 2D and 3D)
  - **How is their fate chosen in a spatial context?**
- And at a single-cell level, each also evolves with time
  - = they do not have a fixed role
    - Directional: matrix-producing  $\rightarrow$  spore-producing or (v rare motile  $\rightarrow$  spore-producing)
    - Bidirectional: motile phase  $\leftrightarrow$  matrix-producing phase
  - **How their fate chosen as time passes?**
- Increasing evidence of stochastic switching (alternative to responsive switching) in maintaining the heterogeneity
  - **Which part allows randomness to play important roles?**
- And why? -what are the importance of coexisting pattern of different subpopulations – at cellular and population level
- To understand the regulation is to understand how their fate is chosen
  - Can influence their behaviors by manipulating genes that play key roles in driving these phenotypic changes
  - Can test for any benefit of these switching behaviors (using evolution/competition experiment)
- Focus : Motile phase  $\longleftrightarrow$  Matrix-producing phase switching regulation



<https://www.biofilms.ac.uk/?s=b+subtilis>

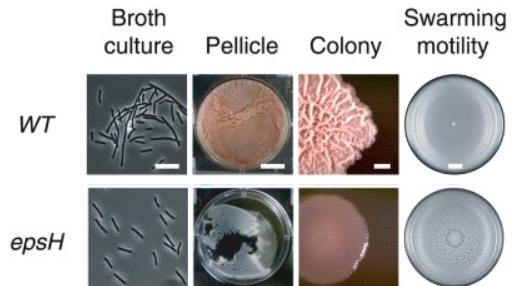
<https://www.nature.com/articles/s41467-020-14431-9>

<https://www.mdpi.com/2076-2607/10/6/1108>

# Target genes of the switching regulation

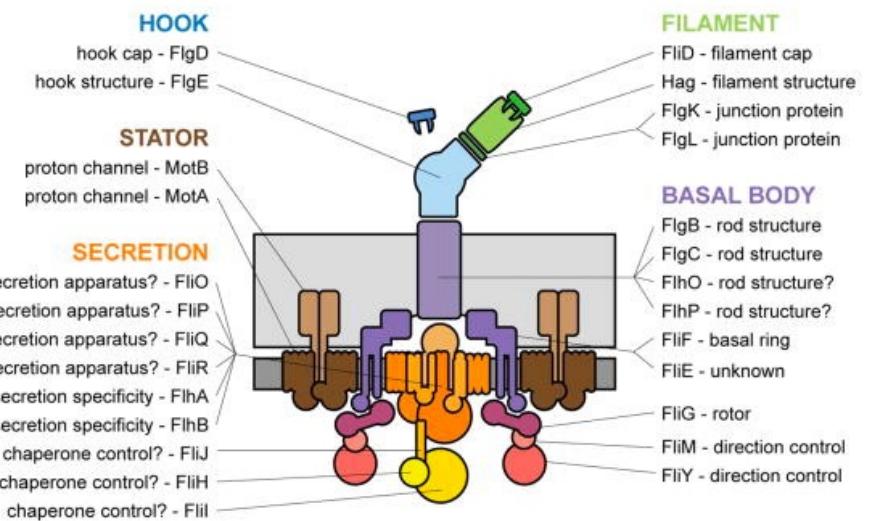
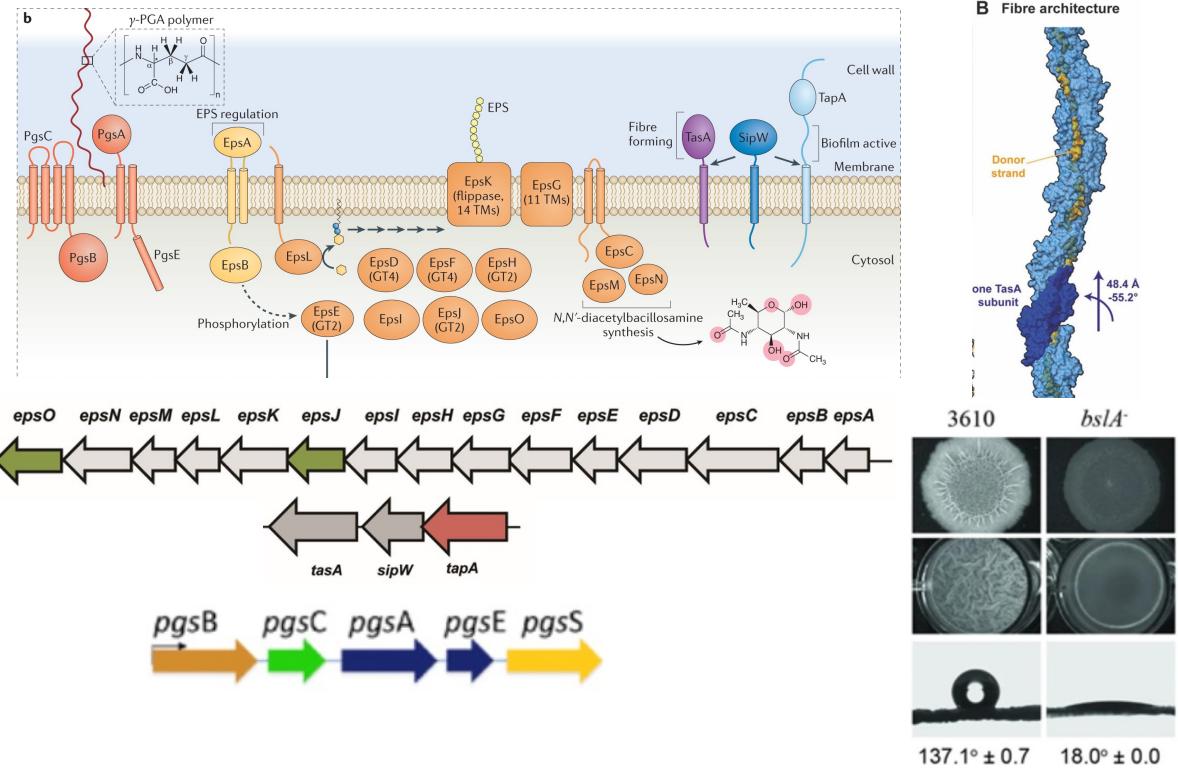
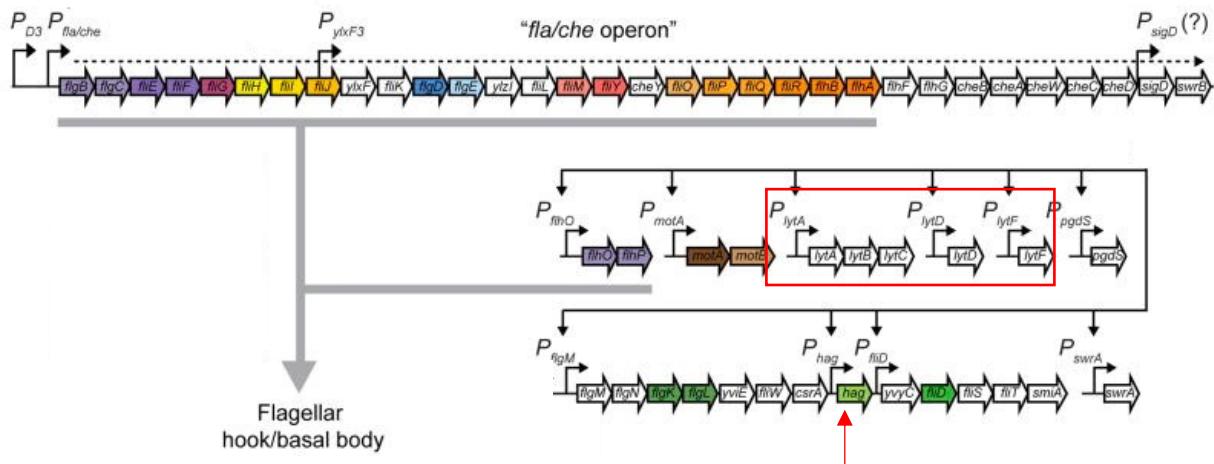
- Matrix genes

- tapA-sipW-tasA* operon  $\Rightarrow$  scaffold proteins
- eps* operon  $\Rightarrow$  Exopolysaccharides
- pgs* operon  $\Rightarrow$  Poly-glutamic acid
- bslA*  $\Rightarrow$  Coating protein



- Motile genes

- hag*  $\Rightarrow$  Flagellin
- lytC,D,F*  $\Rightarrow$  autolysins



<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4869327/>

<https://www.biorxiv.org/content/10.1101/2022.03.14.484220v1.full>

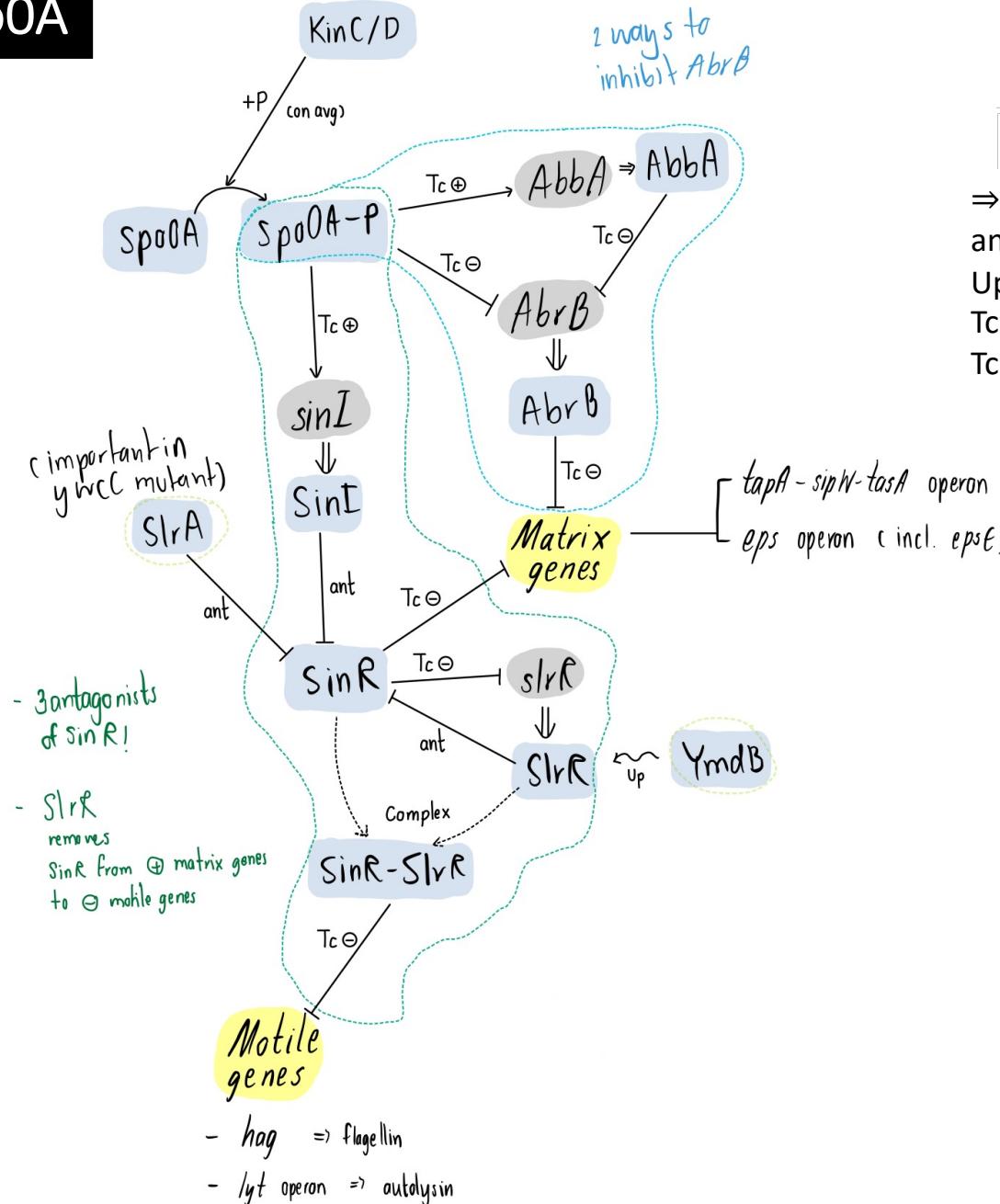
# Modules directly involved with SpoOA

## Module 1: Spo-Abs

- ↑ PP of spoOA
- ↑ Abba level    ↓ AbrB level
- ↓ AbrB level
- ↑ matrix production

## Module 2: Spo-Sins-Slr

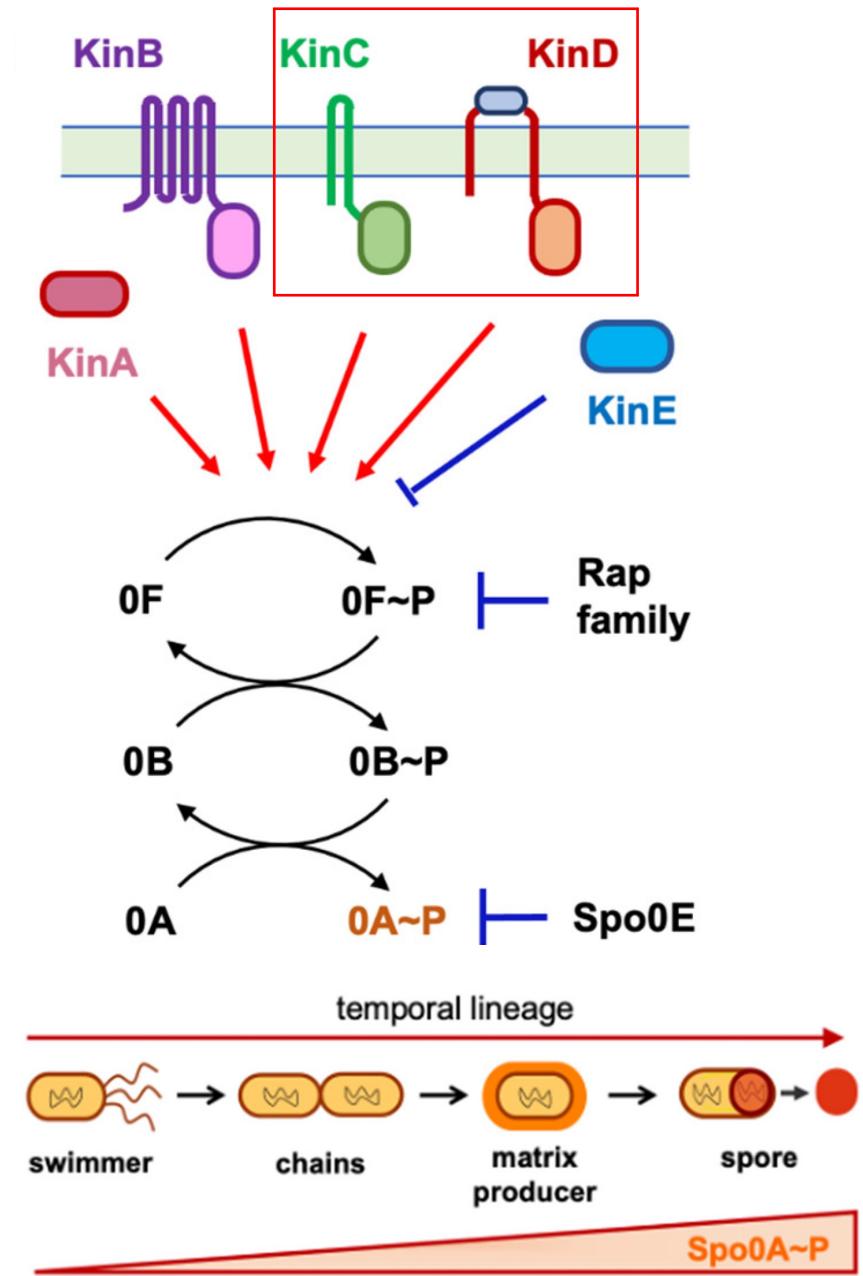
- ↑ PP of spoOA
- ↑ SinI level
- ↑ SinI-SinR    ↓ tetra SinR
- ↓ SinR activity
- ↑ SlrR level (= ↑ YmdB)
- ↑ SinR-SlrR    ↓ tetra SinR
- ↓ motility    ↑ matrix production



Grey = regulatory gene  
 Blue = protein  
 Yellow = target genes  
 ⇒ = encode  
 ant--| = antagonize  
 Up--> = upregulate  
 Tc $\oplus$ --> = transcriptional activation  
 Tc $\ominus$ --| = transcriptional repression

# Details of Spo0A (Stage 0 sporulation protein A)

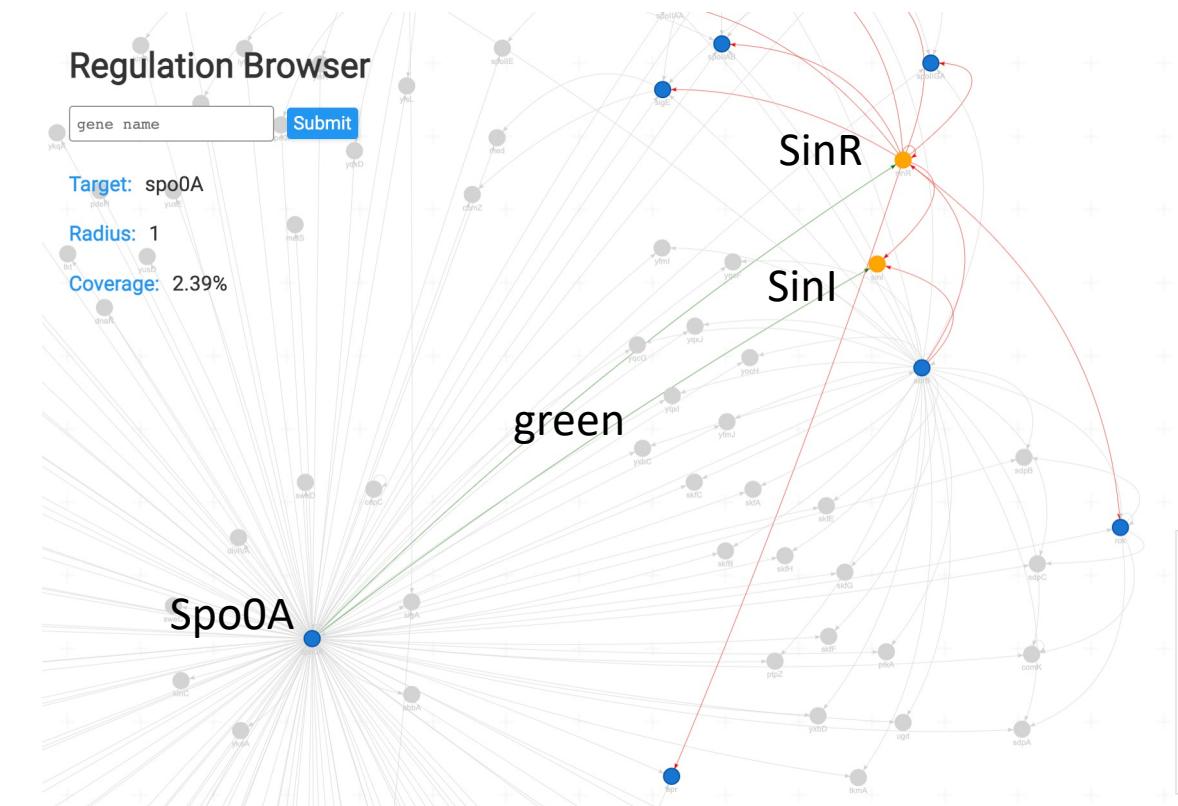
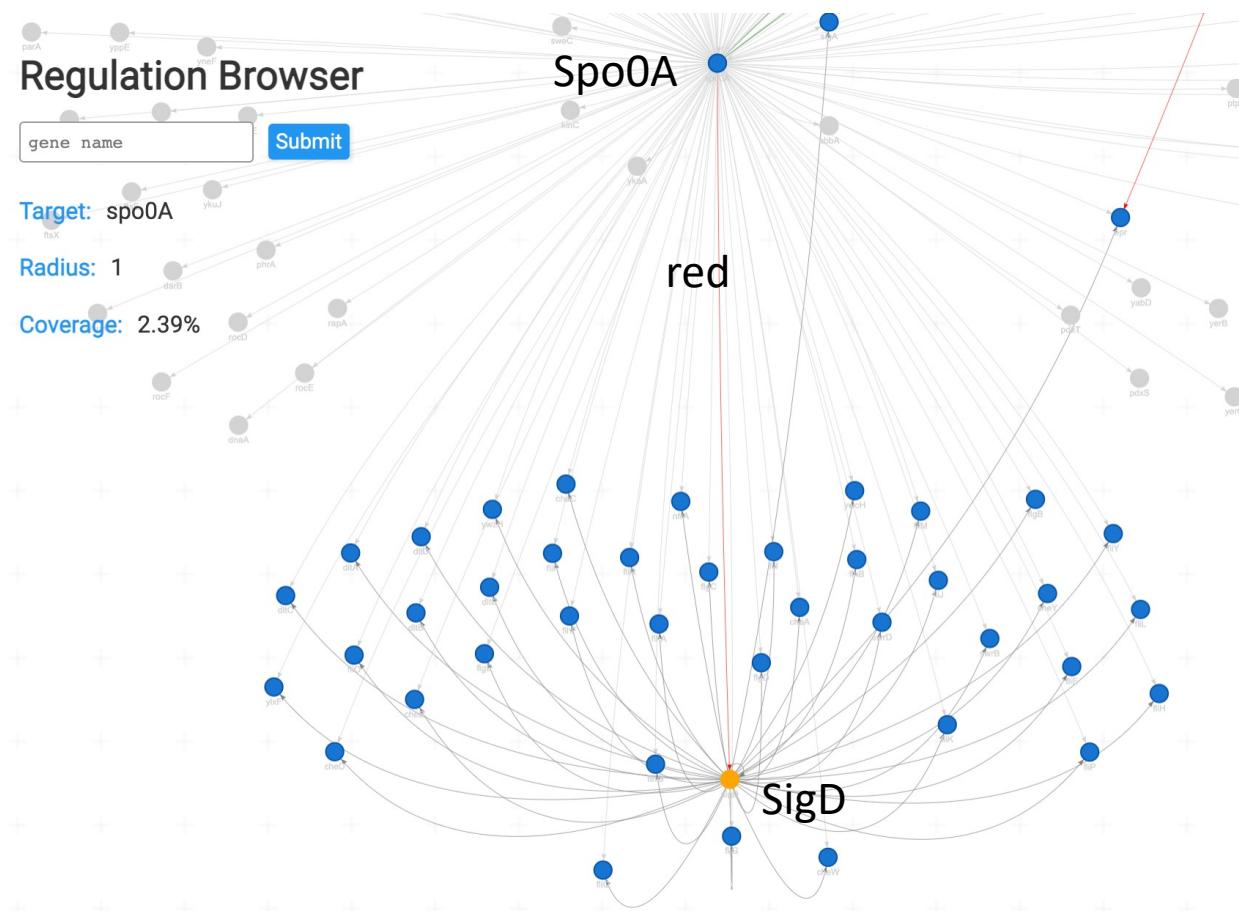
- Spo0A is a crucial regulator
  - integration ‘hub’ for many competing and overlapping signals
    - 100+ target genes – low and high-threshold genes
  - Activation by the phosphorelay from the kinases
    - “No single kinase is solely responsible for matrix gene expression, but rather the contribution of different kinases changes depending on the signals present in the growth conditions being analysed”
  - Initiation of **biofilm** formation is promoted when **moderate** levels of phosphorylated Spo0A are reached within a cell.
- Inhibit matrix production genes (*eps* and *tapA* operon) – 2 main activating pathways
  - Through reduction of AbrB level
  - Through reduction of tetrameric SinR level (via increase of an antagonist SinI level)
- The level of Spo0A-P is not elevated homogeneously in the population
  - Due to noise and positive feedback  
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2279205/#B8>
- Some evidence that it represses the transcription of *sigD* (which means the whole *fla/che* operon?)
  - Cannot find the primary paper
- External input - later



# (cont.) Details of Spo0A (Stage 0 sporulation protein A)

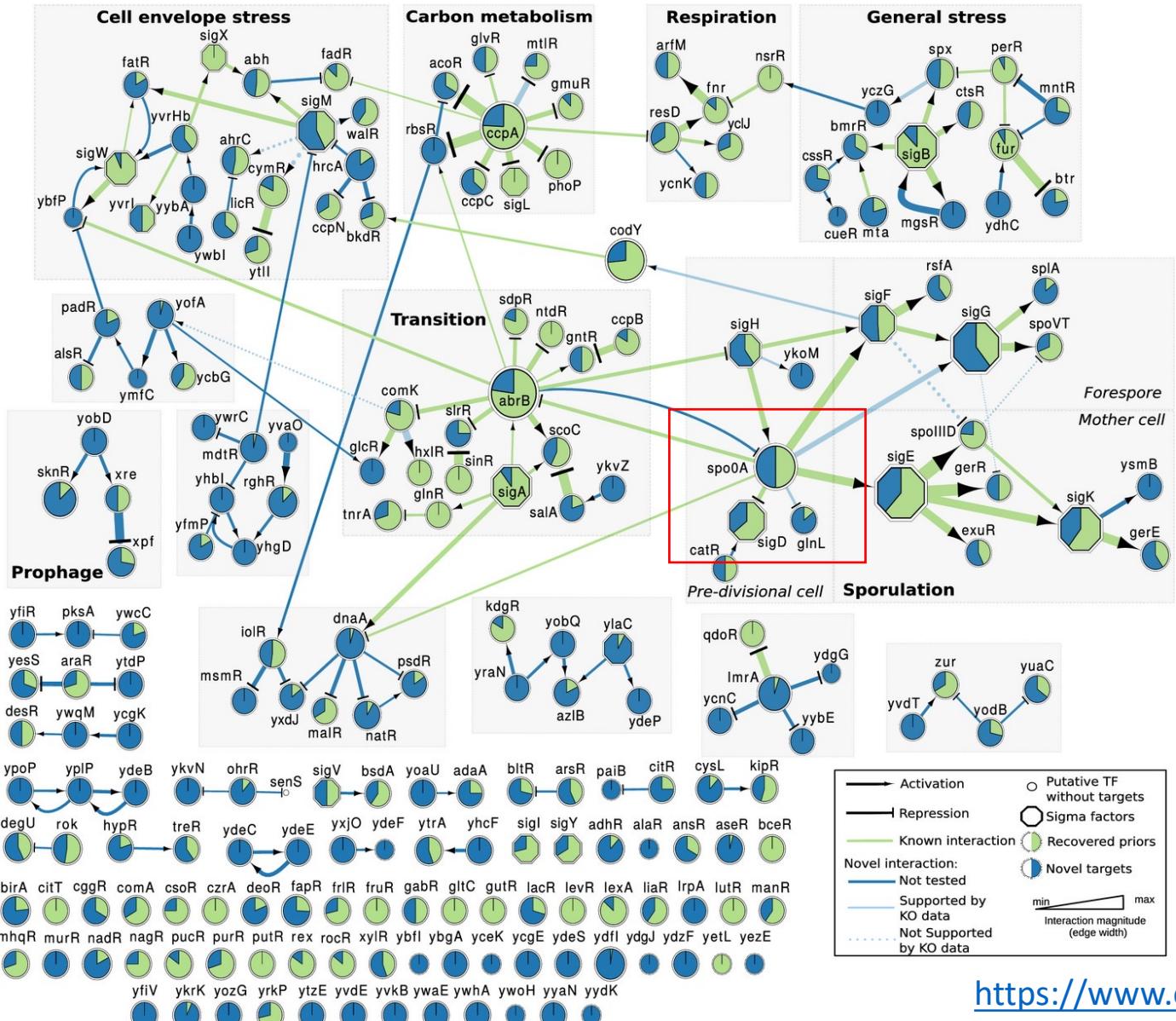
SubtiWiki – suggest Spo0A inhibit sigD – how?

<http://subtiwiki.uni-goettingen.de/v4/regulation?gene=2C54FE2ADC82FF414D732018C90649D477A925AD>

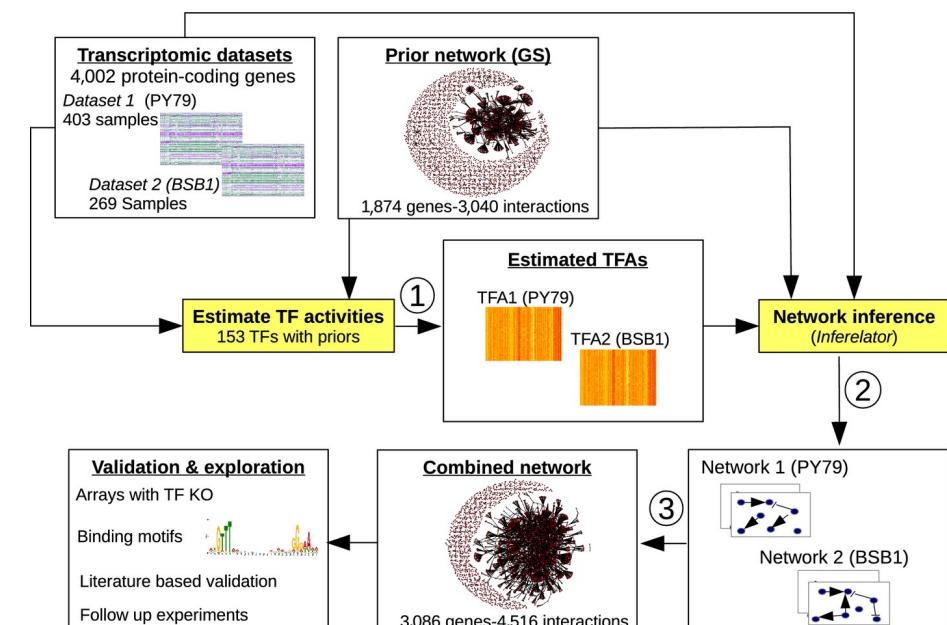


# (cont.) Details of Spo0A (Stage 0 sporulation protein A)

How? – not mechanistically conclusive – only shown in Complex GRNs



$\sigma$  factors (octagons) and other transcription factors (circles).



# Details in SinI-SinR-SlrR interactions

Many mutant papers support their importance in chaining-swimming fate

**Common interpretation:** The double-negative loop is epigenetic in that both the **low-SlrR** and **high-SlrR** states are stable for many generations. During biofilm formation, **SinI** is produced and drives the **switch** into the high-SlrR state by binding to and inhibiting SinR.

<https://www.nature.com/articles/nrmicro2960>

Inconclusive: Does SinR also Tc repress *sinI*?

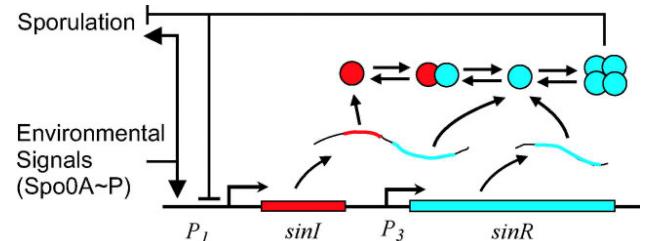
- Many biology and modelling papers ignored it completely
- Origin: P1 and P3 activation and translation of each mRNA seem to depend on growth phase

SinR transcriptionally represses its antagonist SinI **by binding to the *P*<sub>1</sub> promoter** ([SMITH et al. 1991](#)).

- When resource is scarce, spoOA is working to activate *P*<sub>1</sub>.
- **SinI expression is 10-fold greater than that of SinR from *P*<sub>1</sub> mRNA.**

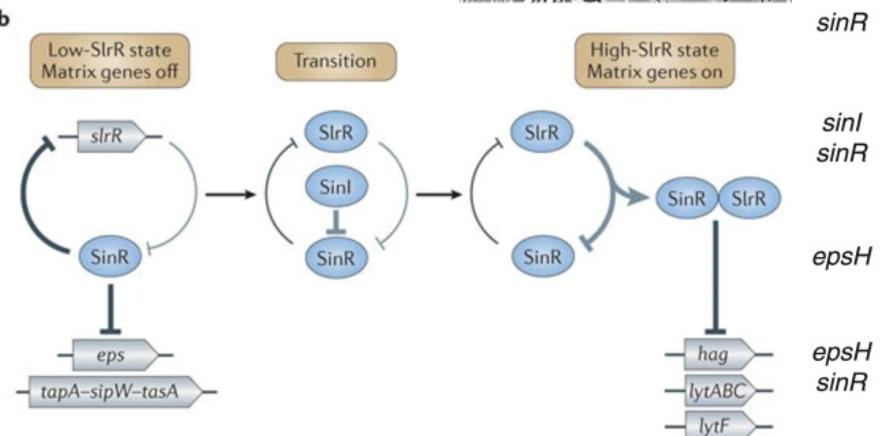
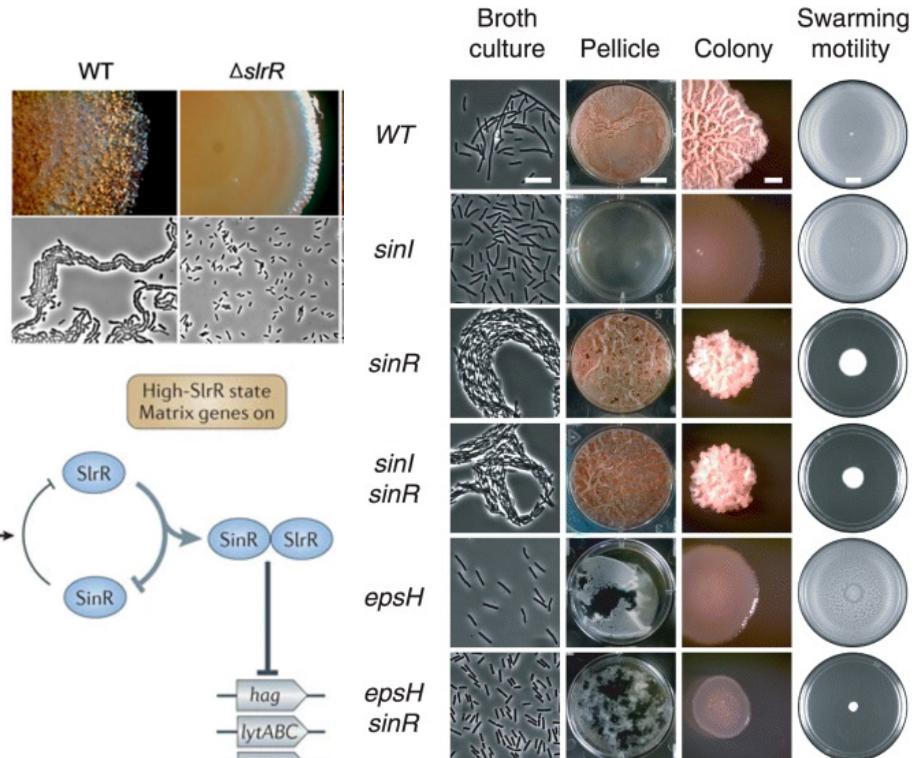
<https://europepmc.org/article/med/15466432> Christopher A Voigt, 2004

When resource is plenty, SinR is expressed constitutively by *P*<sub>3</sub>

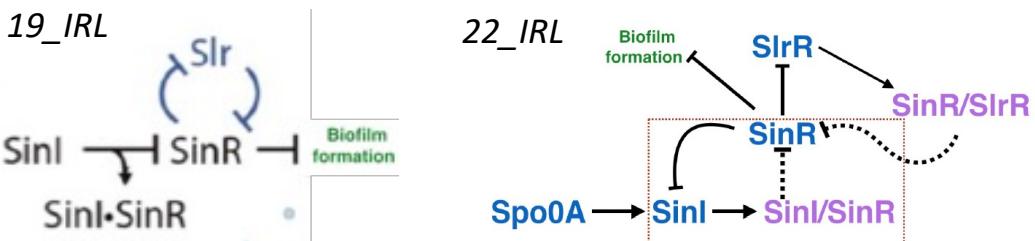
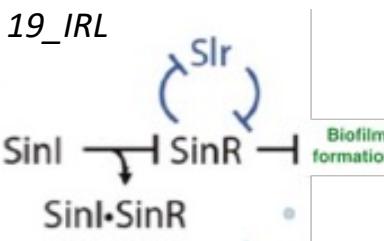


1991

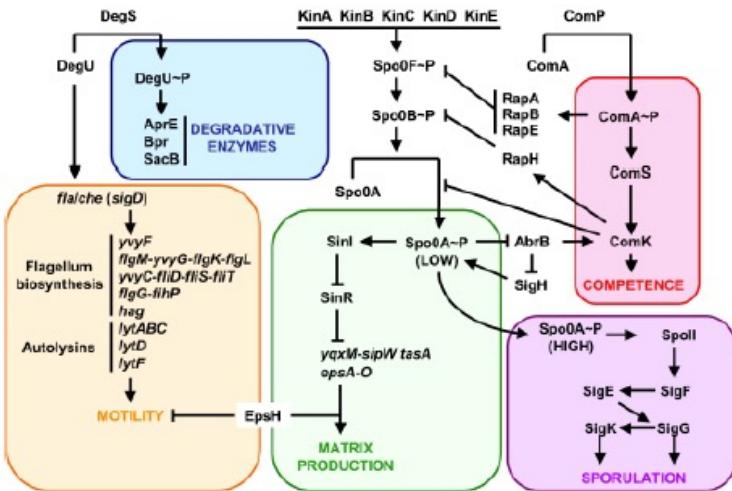
**Sin, another DNA-binding protein, represses the same genes, *spoIIA*, *spoIIIE* and *spoIIIG*, that are activated by SpoOA. Thus sporulation is controlled at the two earliest stages by at least two repressors. Sin and AbpB are repressors of other late-growth functions.**



Two modelling papers (2019, 2022)



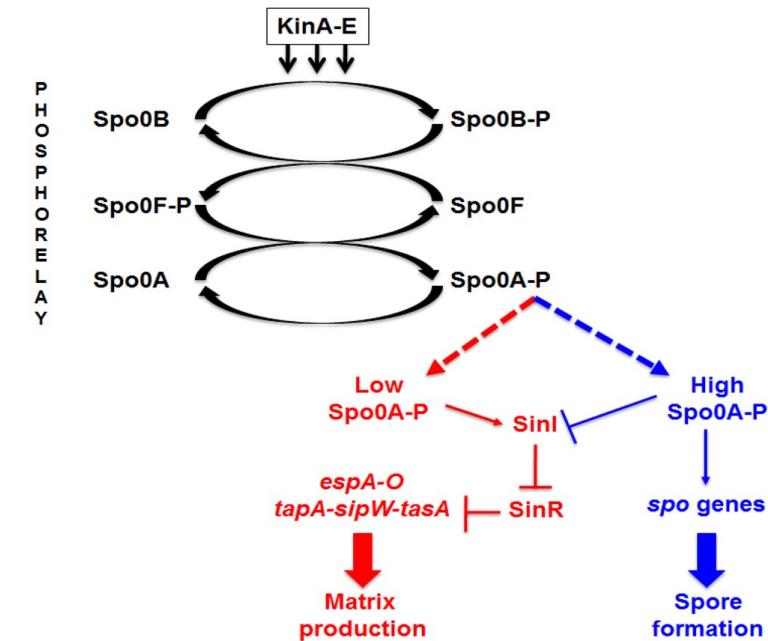
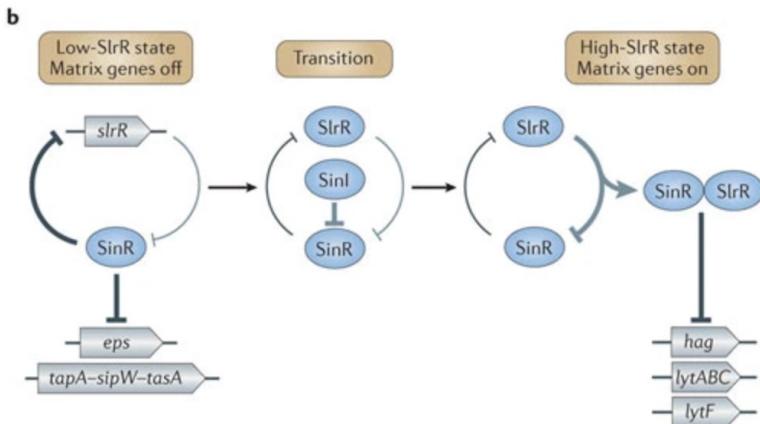
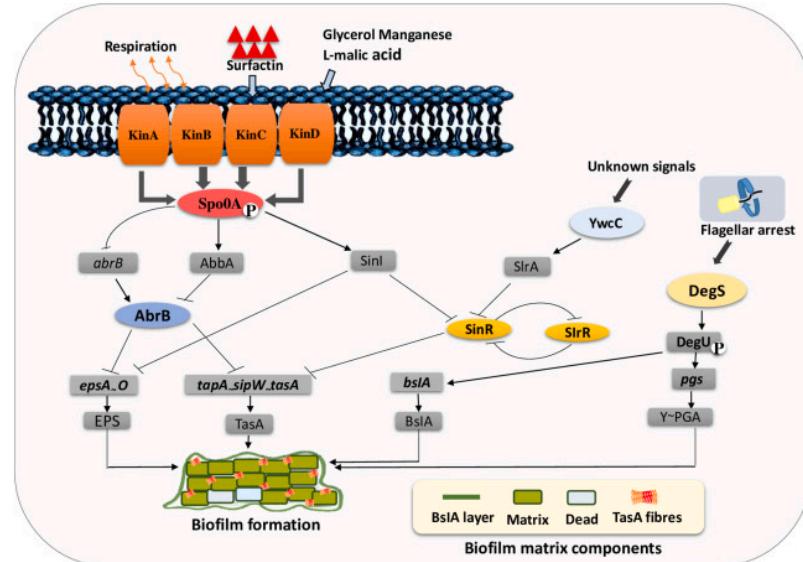
# Most biology papers ignore SinR repression on *sinI*



**Fig. 3.** Network of the different genetics pathways related to cell differentiation in *Bacillus subtilis*. Genes related specifically for each differentiation process are located within the specific frame.

FEMS Microbiol Rev 33 (2009) 152–163

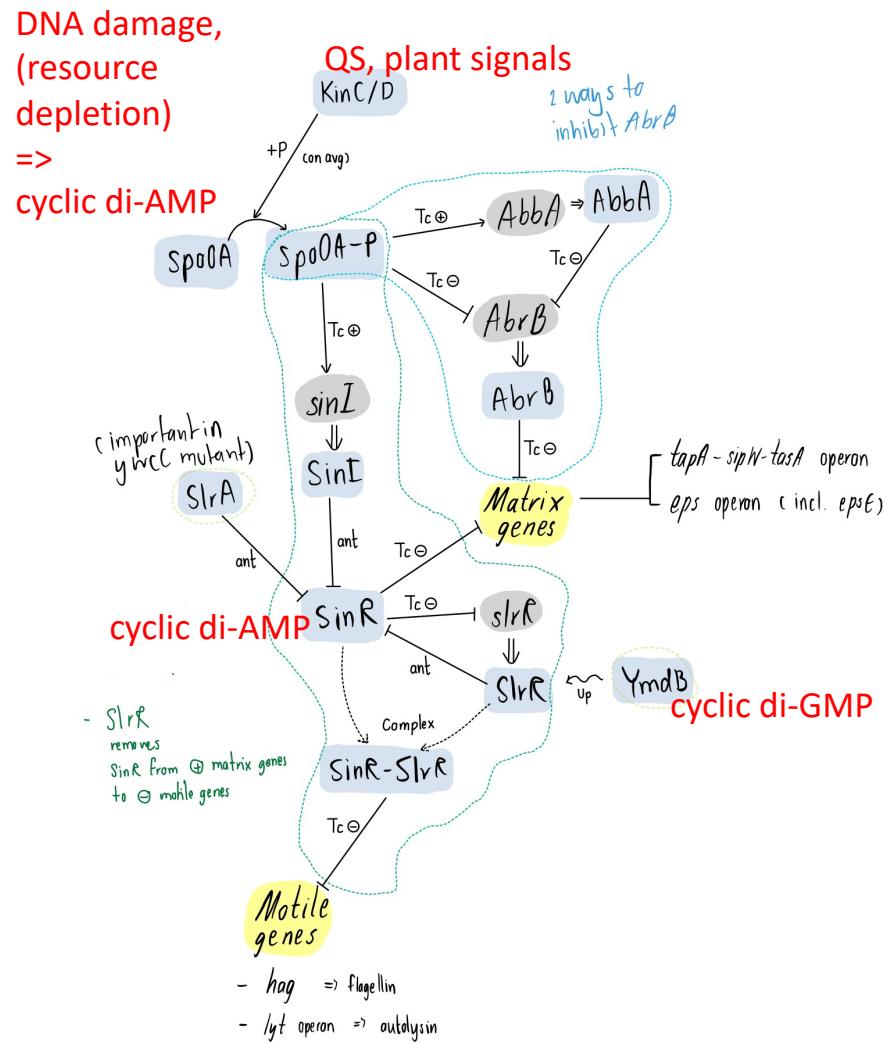
© 2008 Federation of European Microbiological Societies  
Published by Blackwell Publishing Ltd. All rights reserved



# External inputs at Spo0A and Sins-SlrR

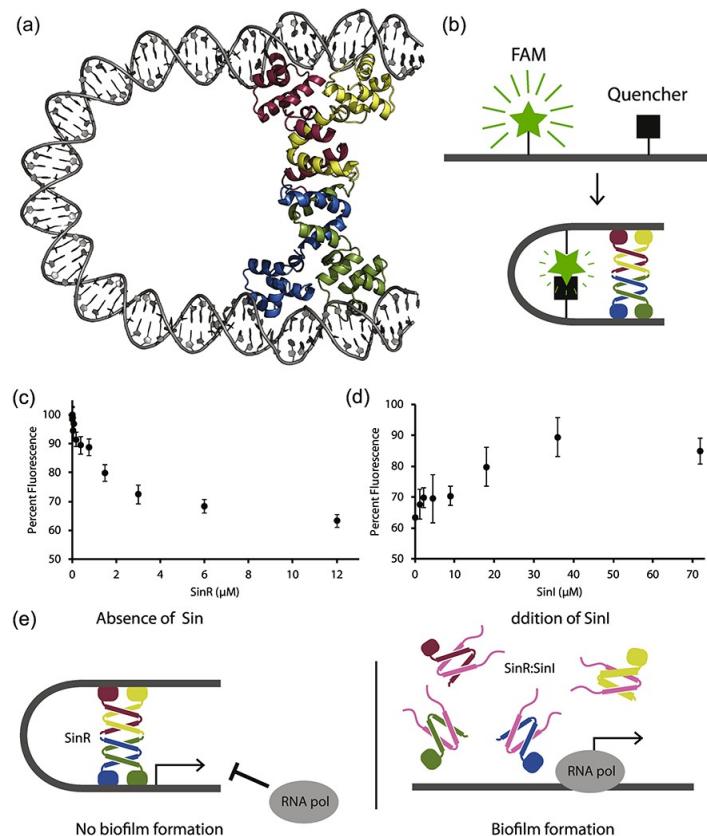
- YmdB on SlrR – regulated by the level of **cyclic di-GMP**?
  - Exp: see a strong reduction of the c-di-GMP levels in *ymdB* mutant.
  - Lit: c-di-GMP is implicated in the control of biofilm formation in many species, but not universally.
  - May only parallels the effects on biofilm formation and motility regulon expression or a causal relation?
- level of **cyclic di-AMP** affect **Spo0A** and **SinR**?
  - Lit: the Spo0A-mediated regulatory pathway is also modulated by the secondary messenger c-di-AMP, a signal for DNA damage
    - No direct evidence\*
  - Exp: increased c-di-AMP reduces expression of the *tapA* and *epsA-O* operons.
    - disruption of *sinR* restores biofilm formation under increased intracellular c-di-AMP
    - c-di-AMP accumulation does not affect the intracellular levels of SinR\*\*
- level of **nutrients** and **QS** affect Spo0A (through Kin or c-di-AMP mediated)?
  - **Resource depletion and high population densities lead to the phosphorylation of Spo0A**
    - this is in the context of a sporulation trigger (possibly through KinA or KinB), but what about the swimming to chaining decision (which should be through KinC or KinD)?
  - Root exudates from tomatoes were shown to trigger biofilm formation in *B. subtilis* in a KinD-dependent manner ([Chen et al., 2012](#)), and also by plant polysaccharides via KinC and KinD ([Beauregard et al., 2013](#)).
  - We see later that KinC is responsible for responding to surfactin and ComX (2 QS signals)
- $\Delta$ Bcspo0A and  $\Delta$ Bcsinl have significantly reduced colonization and nematicidal activity in vitro and biological control efficacy on the tomato plant under greenhouse conditions.

\*the decision to sporulate is a composite consequence of multiple and partially redundant pathways that sense nutrient limitation and cell population density and feed these signals into the phosphorelay and the positive feedback loop that governs the synthesis and phosphorylation of Spo0A. (2005 doi: [10.1101/gad.1335705](https://doi.org/10.1101/gad.1335705))



\*\*How SinR and c-diAMP may interact? And increase SinR activity?

SinR work bind bending DNA, reduce accessibility of RNA pol (at least, for *TapA* operon)



Allosteric regulator?

-> make SinR better bind to DNA target

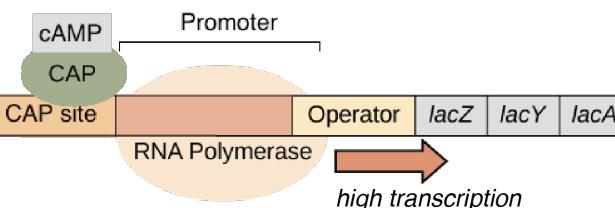
-> additional block of RNAP at this particular site

Recruitment of SinR?

### c-diAMP as a signal of low energy state?

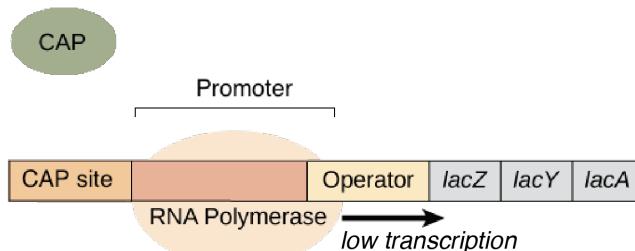
#### Low glucose:

When glucose levels are low, cAMP is produced. The cAMP attaches to CAP, allowing it to bind DNA. CAP helps RNA polymerase bind to the promoter, resulting in high levels of transcription.



#### High glucose:

When glucose levels are high, no cAMP is made. CAP cannot bind DNA without cAMP, so transcription occurs only at a low level.



### Oligomerisation for their function

- Tetramerisation of SinR
- Heterodimerisation of SinR-SinI and SinR-SlrR
- Lord et al., 2019 – can ignore in the model

# Modules indirectly involved with SpoOA

## Module 3

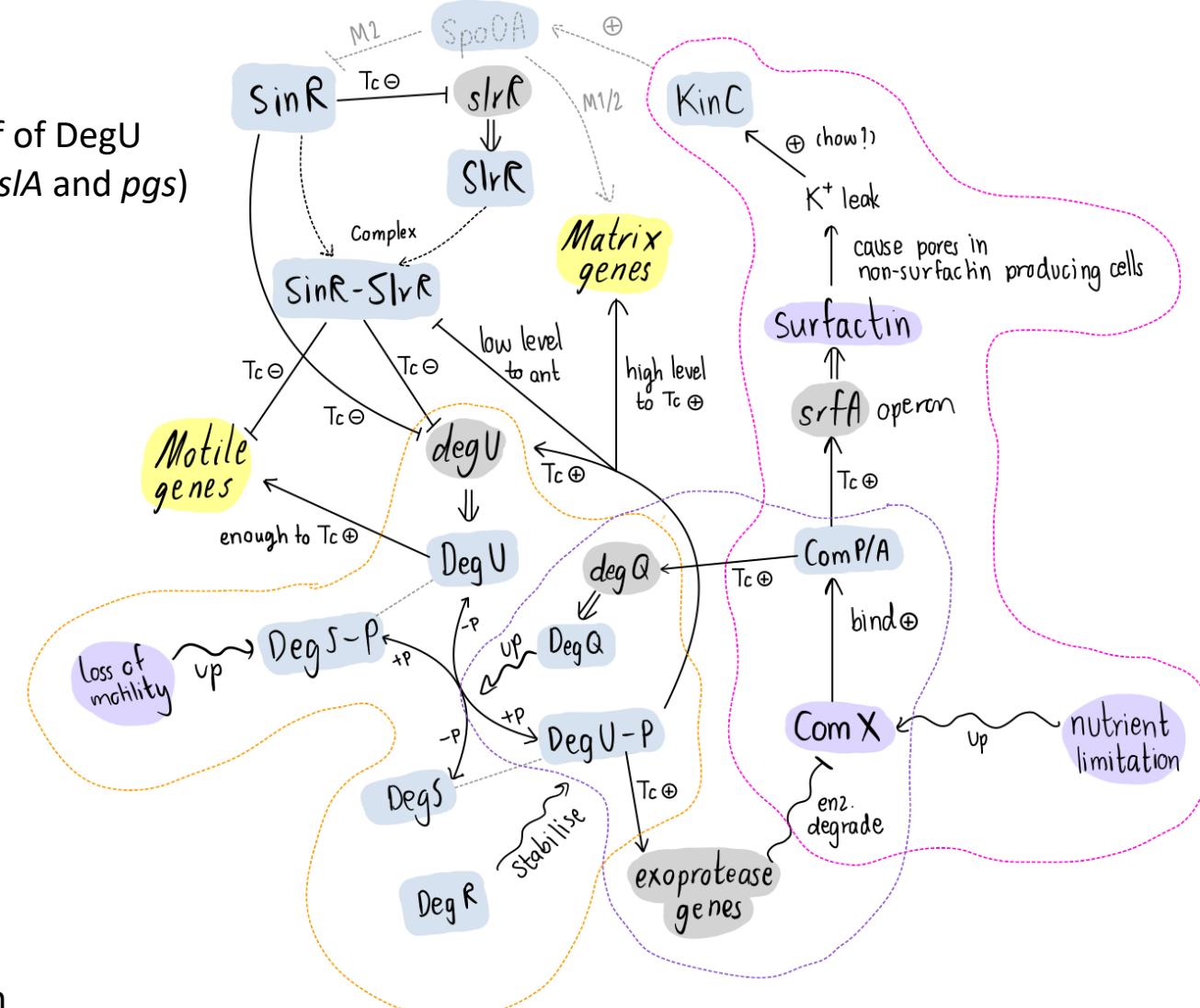
- Begin with loss of motility
- More phosphorylation of DegS -> More phosphorylation of DegU
- Switching from activating motility genes -> matrix genes (*bsIA* and *pgs*)
- & increase DegU production
- Increase matrix and exoprotease production

## Module 4

- Begin with nutrient limitation - ComX signaling activated
- ComX activates ComP/A
- Increase surfactin production & increase ComQ production
- Increase activation of KinC and DegU phosphorylation
- Even more matrix genes activation

## Module 5

- Homeostasis
- Due to the interaction between DegUP and ComX
- Exoproteases degrade ComX
- Less ComP/A activated
- Reduced surfactin production & increase ComQ production
- Possibly keep SpoOA activation in a moderate level

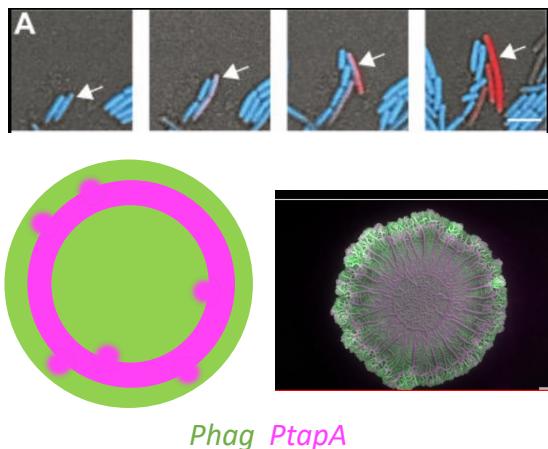


7DEC

## Recap from the first presentation

### 1. Evidence for Motile phase <-> Matrix-producing phase switching behavior

- Spatial pattern in a pop
- Temporal pattern for a cell



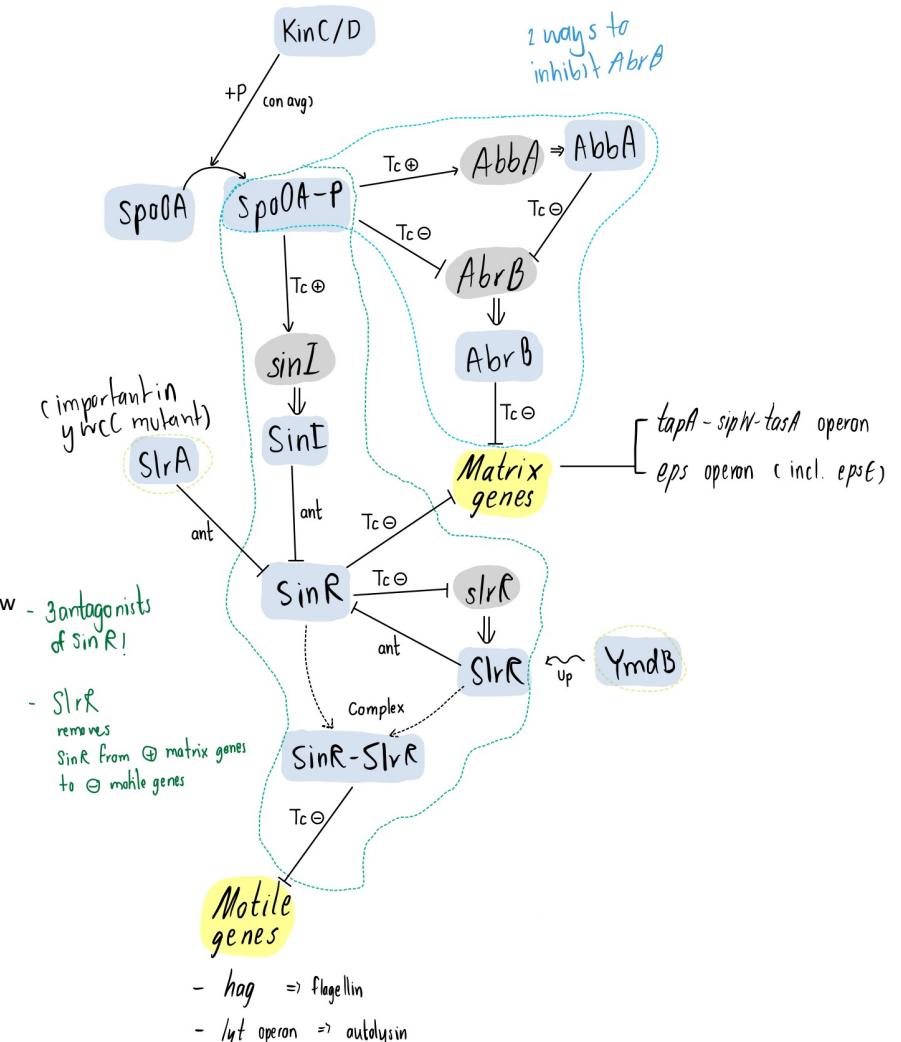
### 2. Introduction to Target genes

Matrix gene	Motile gene
<i>tapA-sipW-tasA</i> operon $\Rightarrow$ scaffold proteins	
<i>eps</i> operon $\Rightarrow$ exopolysaccharides	
<i>pgs</i> operon $\Rightarrow$ Poly-glutamic acid	
<i>bslA</i> $\Rightarrow$ coating protein	
<i>hag</i> $\Rightarrow$ flagellin	
<i>lytC,D,F</i> $\Rightarrow$ autolysins	

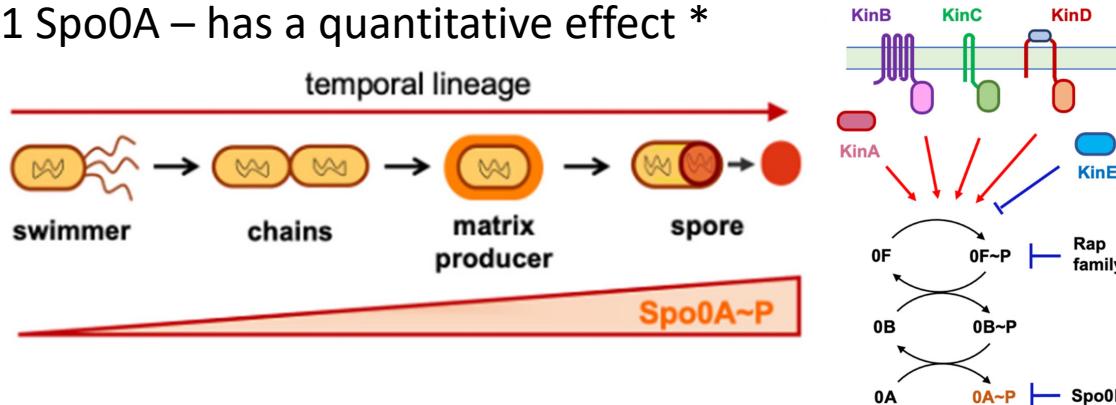
\**EspE* is a clutch to a motor of flagella

### 3. Regulatory network and possible types of interactions

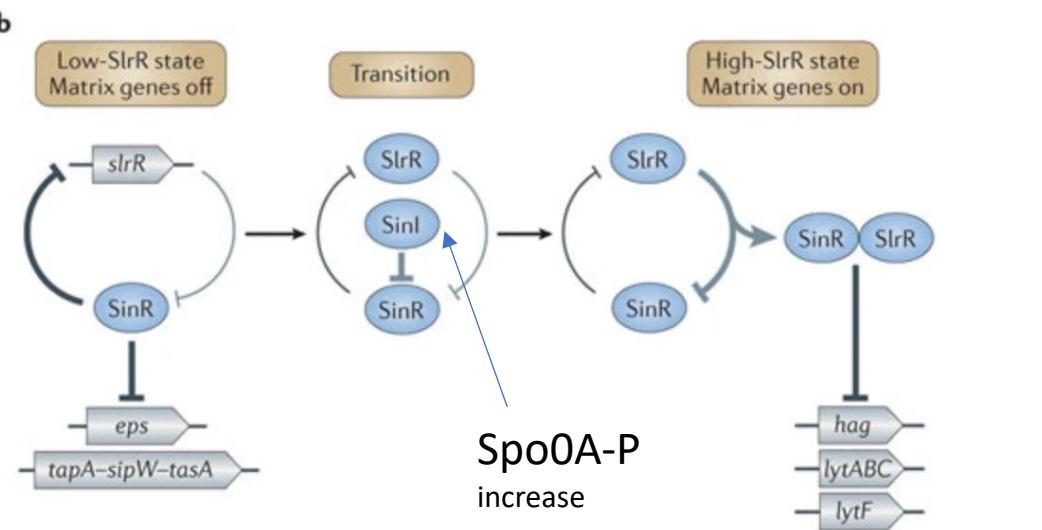
- Directly involved with Spo0A
- Spo0A phosphorylation inhibits matrix production genes (*eps*)  
Through reduction of AbrB level  
Through reduction of tetrameric SinR level (via increase of a



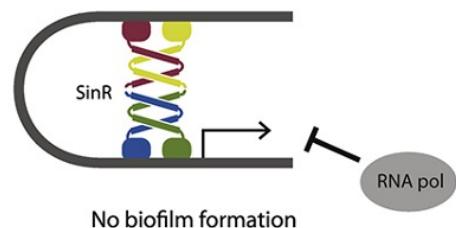
#### 4.1 Spo0A – has a quantitative effect \*



#### 4.2 SinI-SinR-SlrR – bistable – SinI = a switch



#### 4.3 External inputs in Spo0A and SinI-SinR-SlrR



c-di-AMP reduces expression of the *tapA* operons through increasing SinR activity

#### Suggested experiment

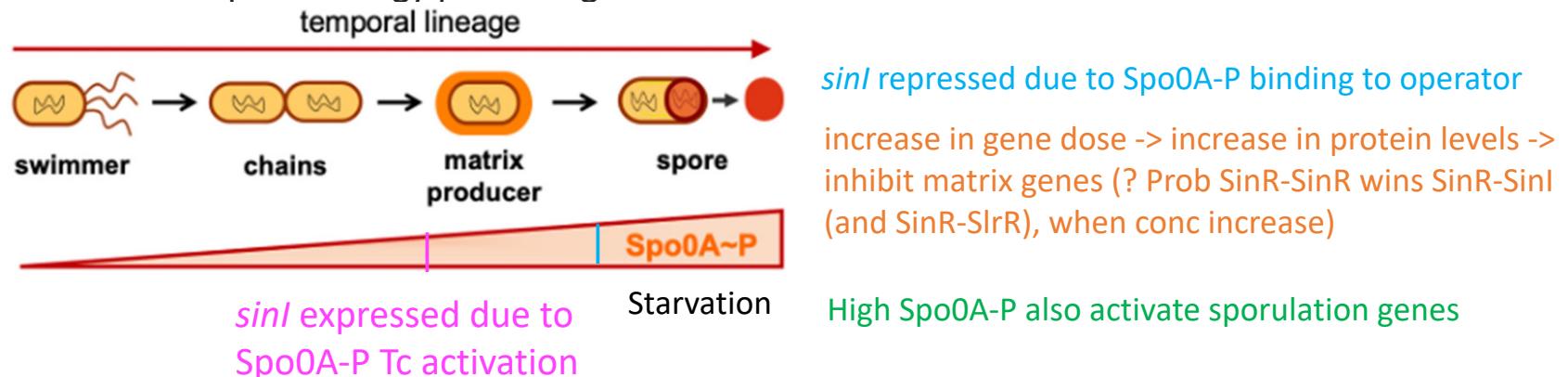
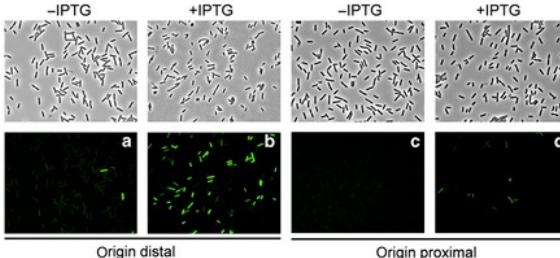
- Frequency of switching in growing biofilms
  - Microscopy problem
- Spo0A
  - Tc repress *sigD* or antagonize SigD?
  - How is it get increasingly phosphorylated under starvation?
    - Reduced N or C signals responsible for its increase
    - Check all kinases and Spo proteins
- Imposing c-di-AMP on cells, will we see less switching to matrix
  - Is c-di-AMP intrinsically formed?
  - Any changes in SinR structure/binding affinity with increased c-di-AMP exposure
  - Do the same with c-di-GMP
- Does SinR back tc repress SinI in our strain in exp conditions

## \* Quantitative effects of Spo0A and Sins on matrix gene activation

- Rate of Spo0A-P level increase determine the **duration** of matrix gene expression in these cells.
  - The promoter of *sinl* contains both a high-affinity activator and multiple low-affinity operators for Spo0A-P.
  - At relatively low Spo0A-P levels - the **high-affinity activator is bound and *sinl* is expressed**.
  - As the levels of Spo0A-P increase - the **low-affinity operators are also occupied and further *sinl* expression is repressed => matrix genes become now inhibited by SinR**

OA consensus sequence (5'-TGTGAA-3')

- The functions of Sinl and SinR are remarkably sensitive to gene **dose**:
  - **doubling** of the *sinl* and *sinR* genes **completely inhibit matrix production**.
  - in early **sporulation**, the presence of **two copies of the chromosome in the mother cell is prolonged**, resulting in higher levels of Sinl and SinR, which is sufficient to **inhibit matrix gene expression**.
- Together, Spo0A-P affinity for the *sinl* promoter and gene copy number of *sinl* and *sinR* ensures that matrix gene expression is transient and that sporulating cells do not expend energy producing extracellular matrix.



# Details of KinC

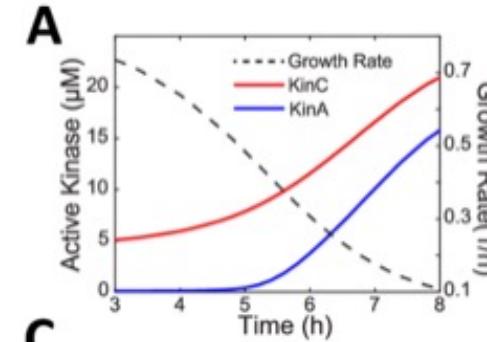
## Context of matrix -> sporulation phase

<https://journals.asm.org/doi/full/10.1128/mbio.01694-21>

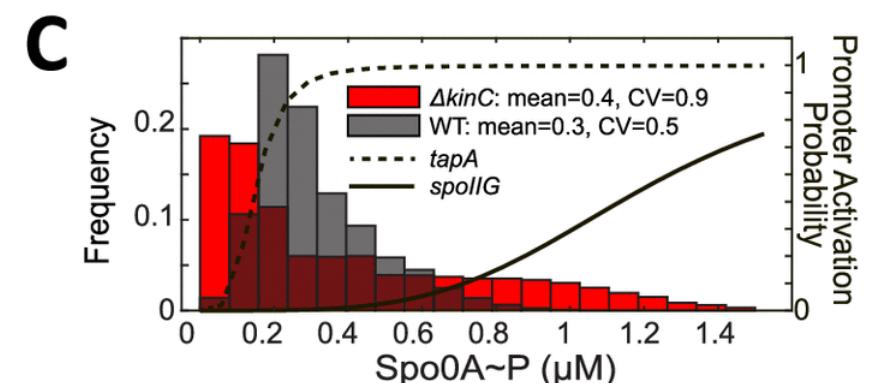
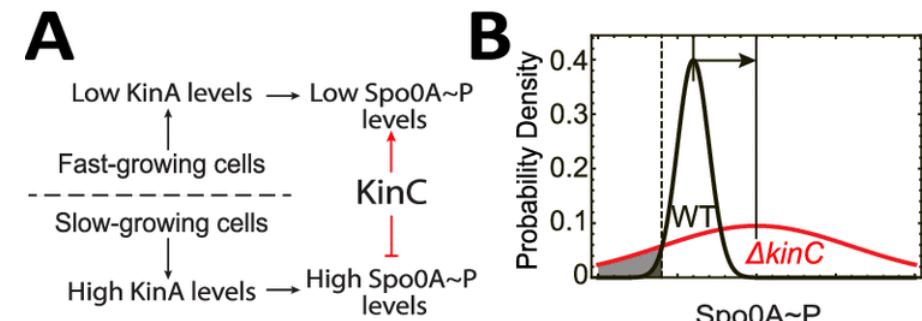
- KinC activates biofilm formation by controlling the **fraction** of cells activating biofilm gene expression.
  - KinC sometimes downregulates sporulation genes, suggesting that KinC has a negative effect on Spo0A activity.
  - To explain: a mathematical model of the phosphorelay
    - KinC has distinct effects on Spo0A at different growth stages:
    - during **fast** growth, KinC acts as a phosphate **source** and activates Spo0A
    - during **slow** growth, KinC becomes a phosphate **sink** and contributes to decreasing Spo0A activity. However, under these conditions, KinC can still increase the population-mean biofilm matrix production activity.
- In a population, individual cells grow at different rates, and KinC would increase the Spo0A activity in the fast-growing cells but reduce the Spo0A activity in the slow-growing cells.
  - This mechanism **reduces single-cell heterogeneity of Spo0A activity**, thereby **increasing the fraction of cells that activate biofilm matrix production**.
  - = more of them have **moderate Spo0A-P level**

### Slow growth rate

- Increase gene dose -> block matrix
- Allow KinC to balance pop -> matrix increase



(B) The gray area indicates the fraction of cells not expressing *tapA*.



(C) Predicted distribution of Spo0A-P level in  $\Delta\text{kinC}$  and WT strains. The solid and dashed lines show how activities of *PspolIG* and *PtapA*, respectively, depend on Spo0A-P level

# Modules indirectly involved with SpoOA

## Module 3

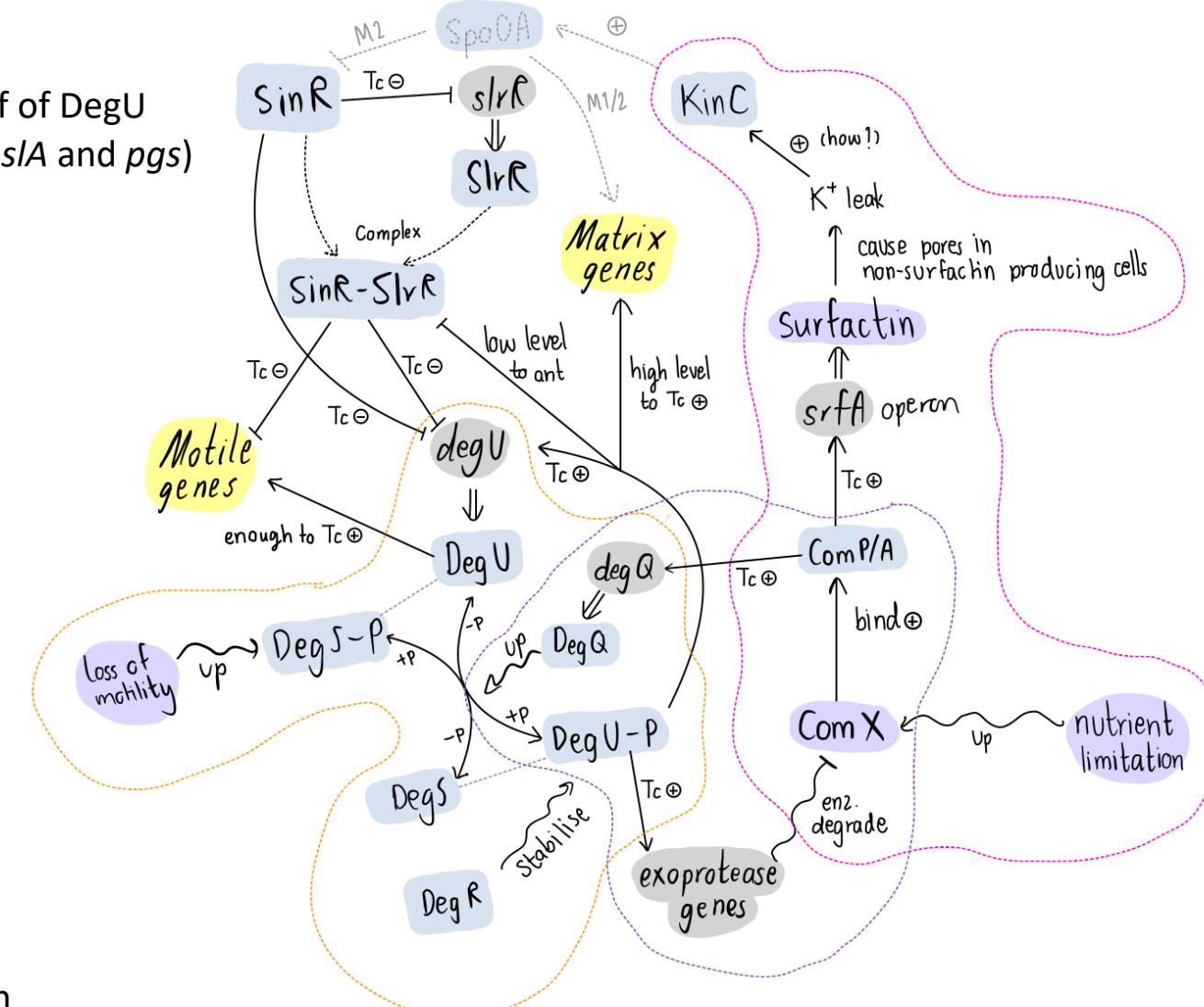
- Begin with loss of motility
- More phosphorylation of DegS -> More phosphorylation of DegU
- Switching from activating motility genes -> matrix genes (*bsIA* and *pgs*)
- & increase DegU production
- Increase matrix and exoprotease production

## Module 4

- Begin with nutrient limitation - ComX signaling activated
- ComX activates ComP/A
- Increase surfactin production & increase ComQ production
- Increase activation of KinC and DegU phosphorylation
- Even more matrix genes activation

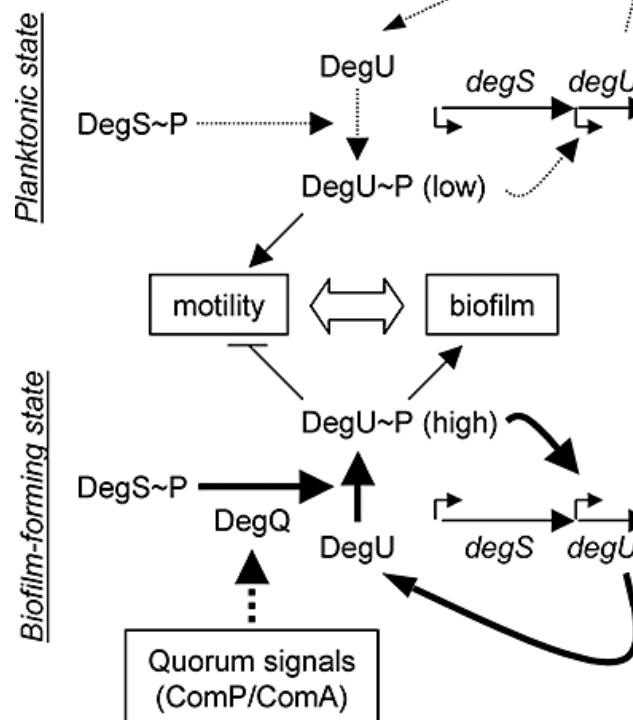
## Module 5

- Homeostasis
- Due to the interaction between DegUP and ComX
- Exoproteases degrade ComX
- Less ComP/A activated
- Reduced surfactin production & increase ComQ production
- Possibly keep SpoOA activation in a moderate level

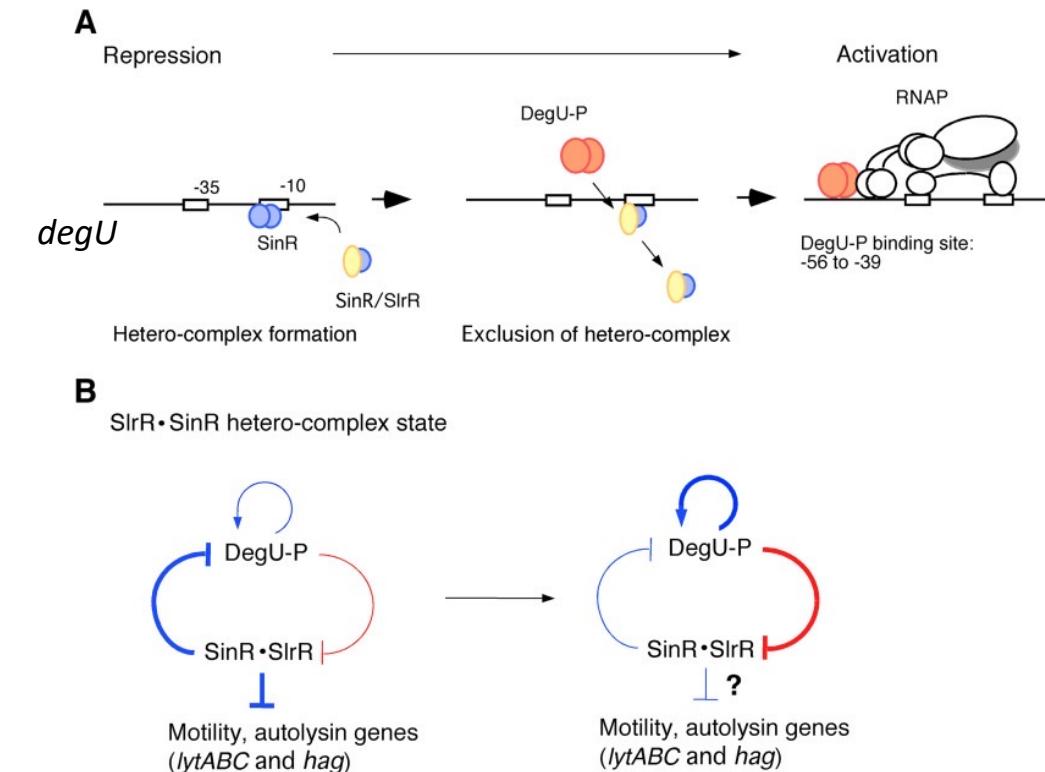


# Details of Deg systems

- *degU* mutation prevents both flagellum formation and biofilm formation
- A low concentration of DegU-P is sufficient to activate transcription of flagellar genes, but a higher concentration is required for transcription of other genes.
- How?
  - DegU-P binds the promoter region of *flgB* with a high affinity
  - But DegU-P binds to the promoter of other DegU-regulated genes with a low affinity and in a DegS-dependent manner.



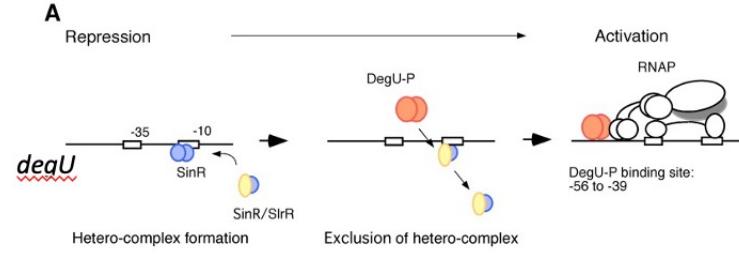
- SinR tetramer and SinR/SlrR dimer can repress *degU*
- DegU-P can displace the SinR/SlrR complex, but not SinR tetramer from the *degU* promoter
  - Maybe DegU-P interacts with SlrR



<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3911177/>

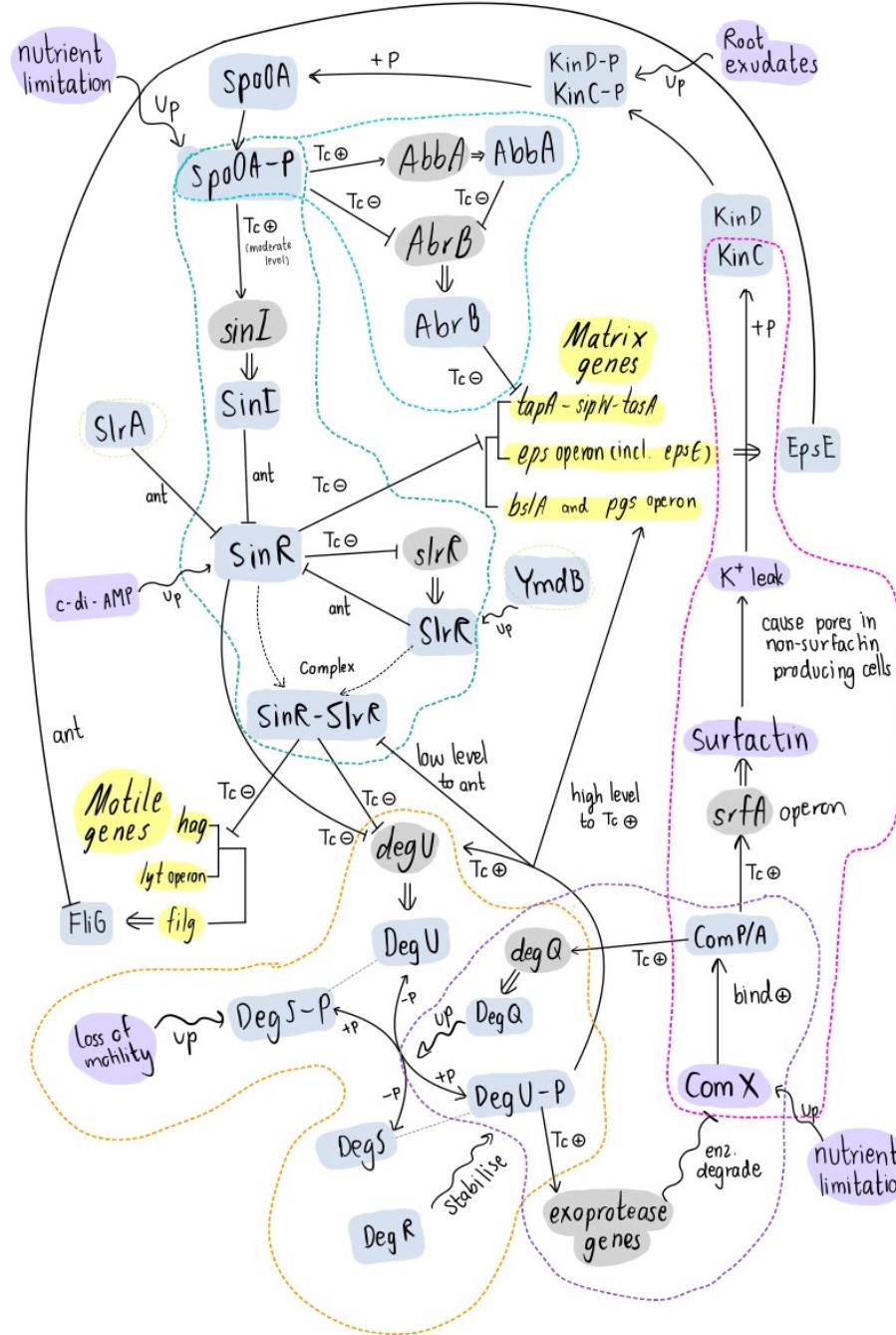
<https://pubmed.ncbi.nlm.nih.gov/17850253/>

14DEC



### Module 3

- Begin with loss of motility
- More phosphorylation of DegS → More phosphorylation of DegU
- Switching from high DegU activating motility genes to high DegU-P activating matrix genes (*bslA* and *pgs*)
- & increase DegU production
- Increase matrix production



### Module 4

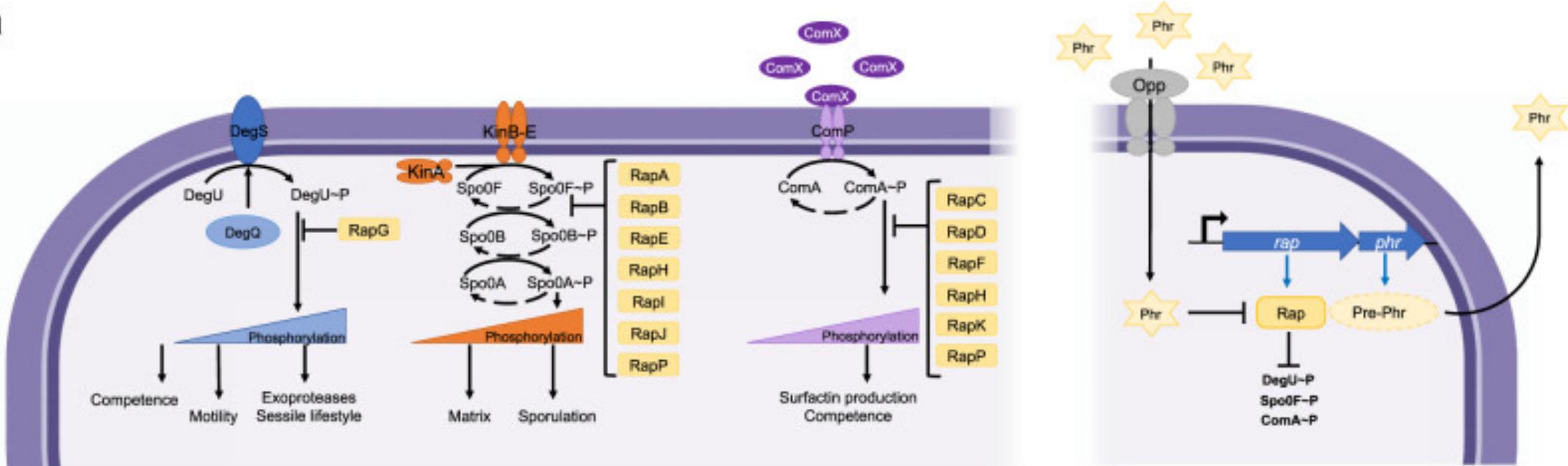
- Begin with nutrient limitation - ComX signaling activated
- ComX activates ComP/A
- Increase surfactin production & increase ComQ production
- Increase activation of KinC & DegU phosphorylation
- Even more matrix genes activation (through KinC and M3)

# External inputs to all three master regulators: Spo0A, DegS/U, ComP/A

Rap inhibits phosphorylation in master regulator

- In strain 3610 - 11 *rap* genes encoded in its genome (*rapA–rapK*) + 1 in pBS32 plasmid
- Work as a Rap–Phr system (*rapP–phrP*)
- Phr = penta/hexapeptides = QS molecules that inhibit Rap

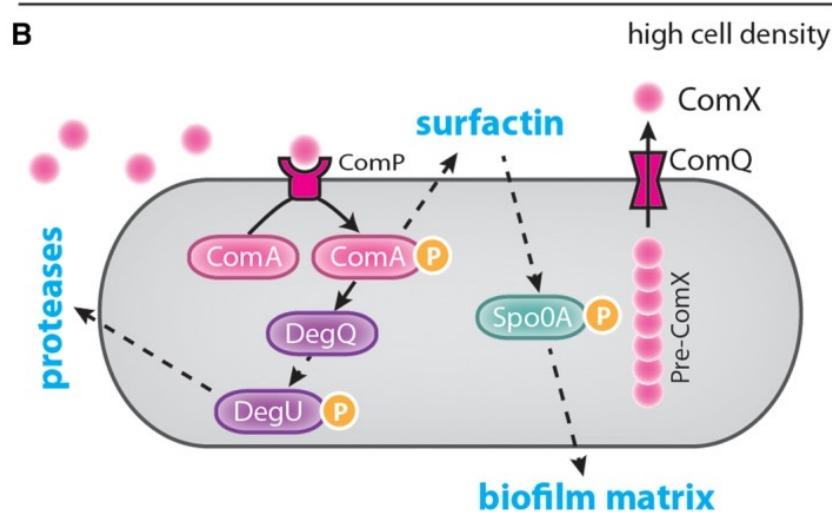
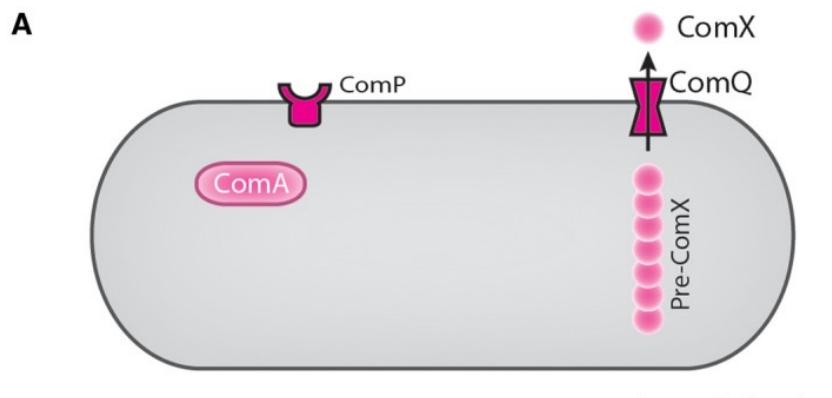
a



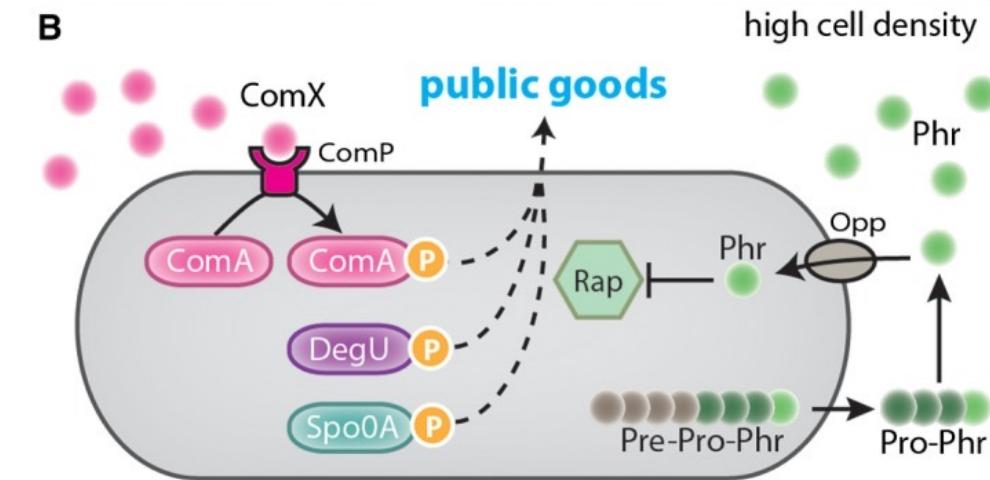
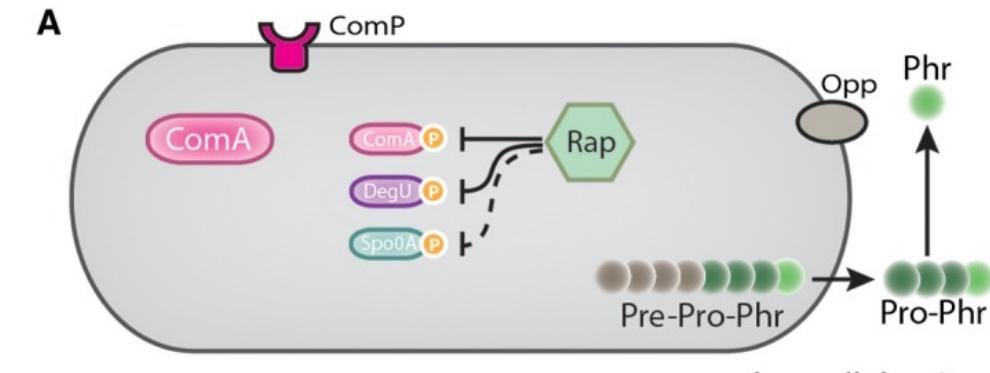
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8044106/>

Competition exp: The differences in the variance of surviving *rap–phr* strains between 2- and 5-day culture conditions, and between planktonic- and pellicle-forming conditions points to dissimilar selection pressures generated by these growth conditions that further reveal the role that Rap–Phr systems play in **environmental adaptability**

(cont) External inputs to all three master regulators

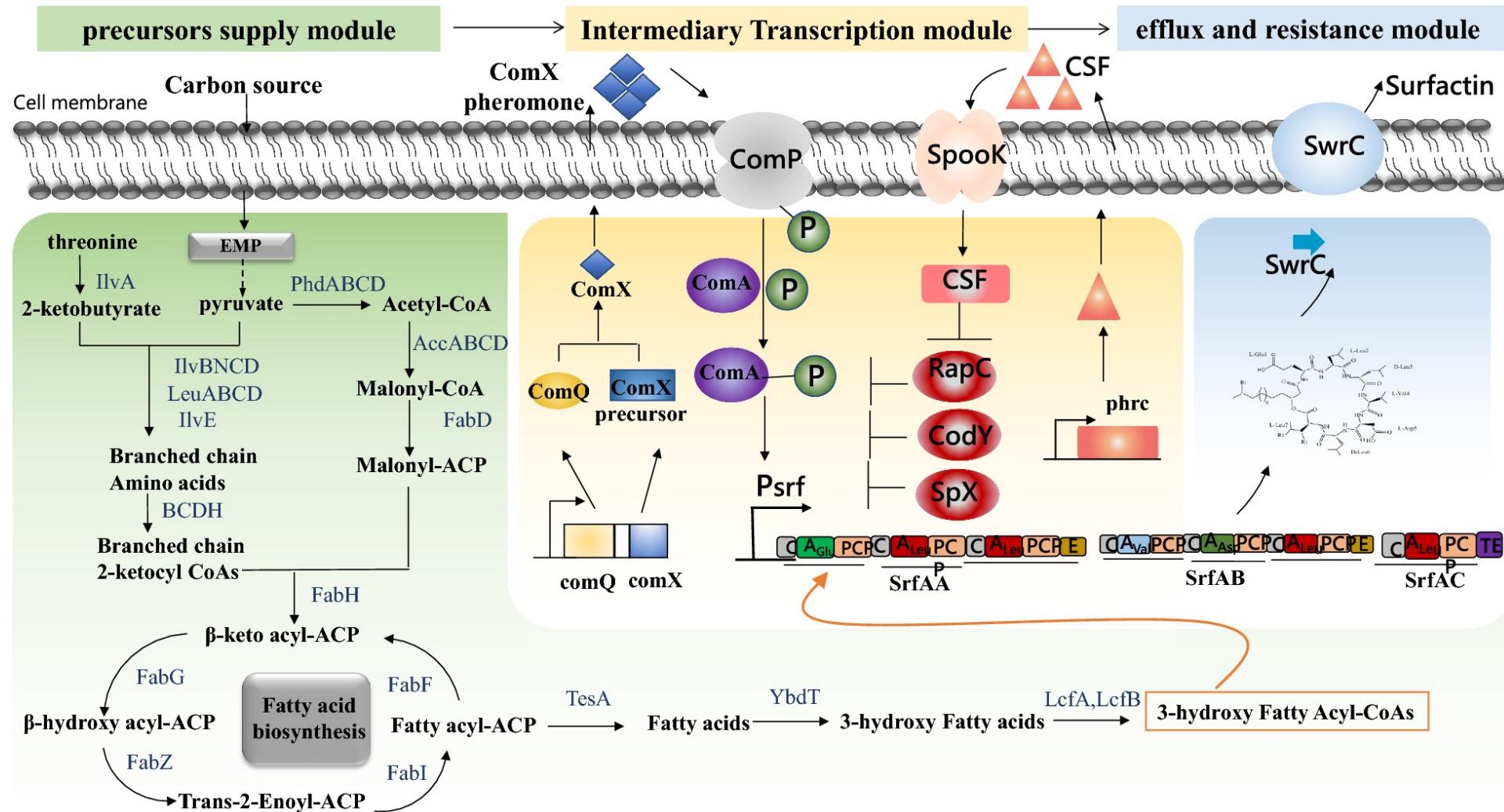


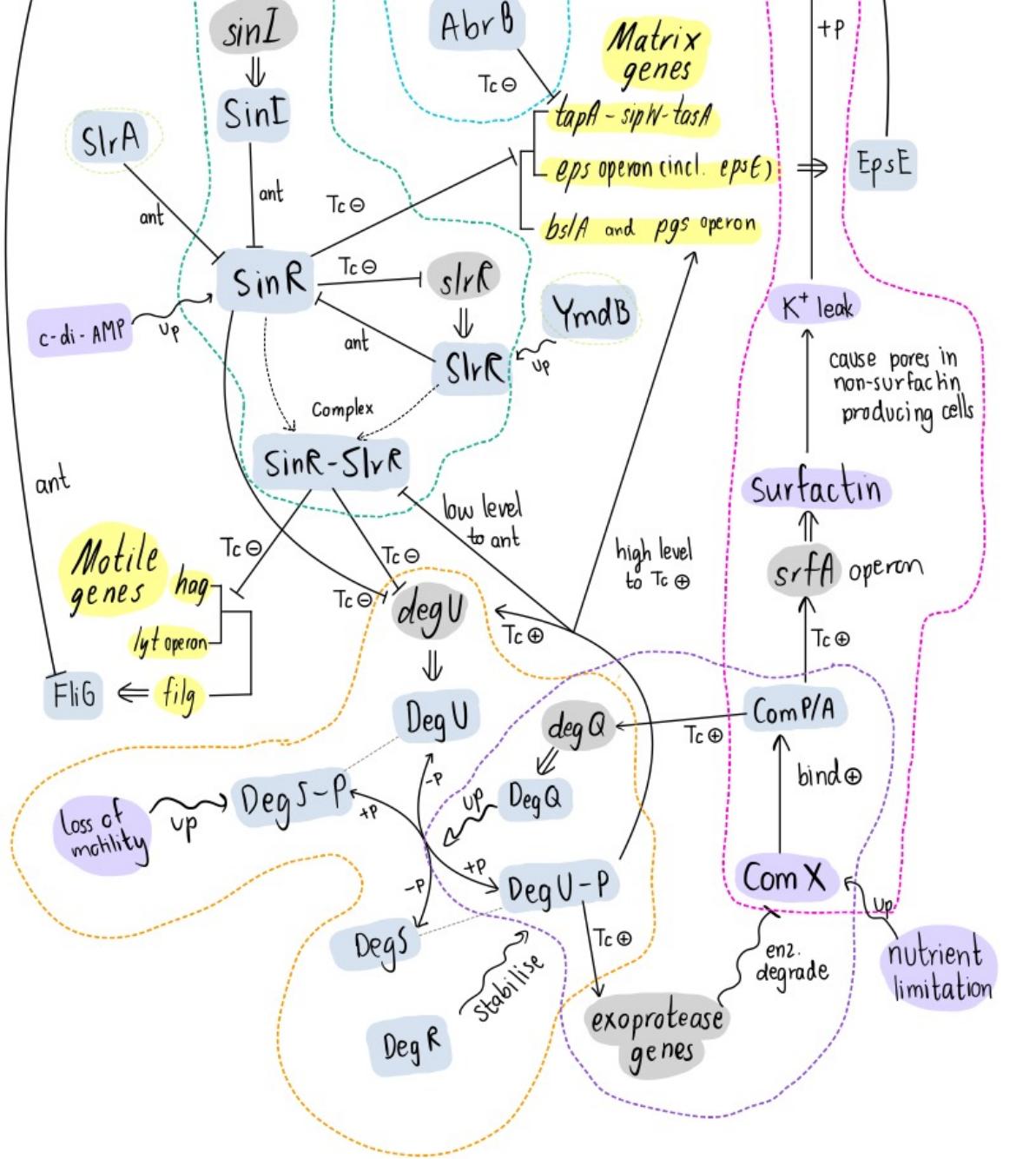
For matrix production



For public good

# ComX + Surfactin working together





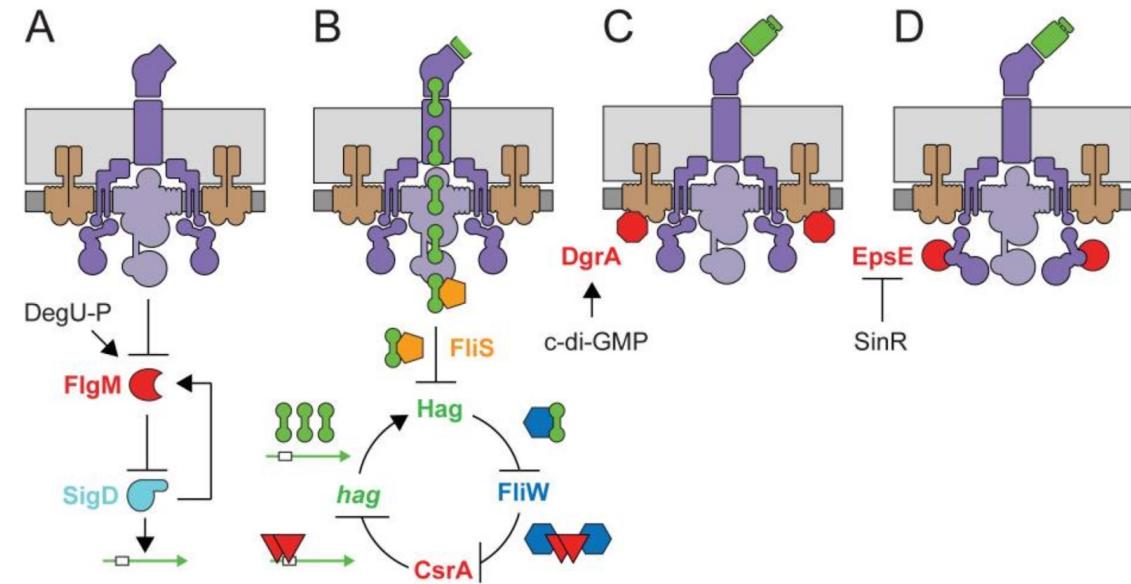
## Module 5

- Homeostasis
- Due to the interaction between DegUP and ComX
- Exoproteases degrade ComX
- Less ComP/A activated
- Reduced surfactin production
  - & less DegQ production
- Reduced KinC activation
  - & less DegUP
- Possibly keep SpoOA activation in a moderate level

# Some genes involved in both matrix production and motility

**EpsE** – bifunctional - independently

- the glycosyltransferase activity that is required for EPS synthesis
- a molecular clutch that inhibits flagellar rotation by interacting with the flagellar motor switch protein, FliG
  - Increase DegS-P?



**TasA** may be necessary for motility activation

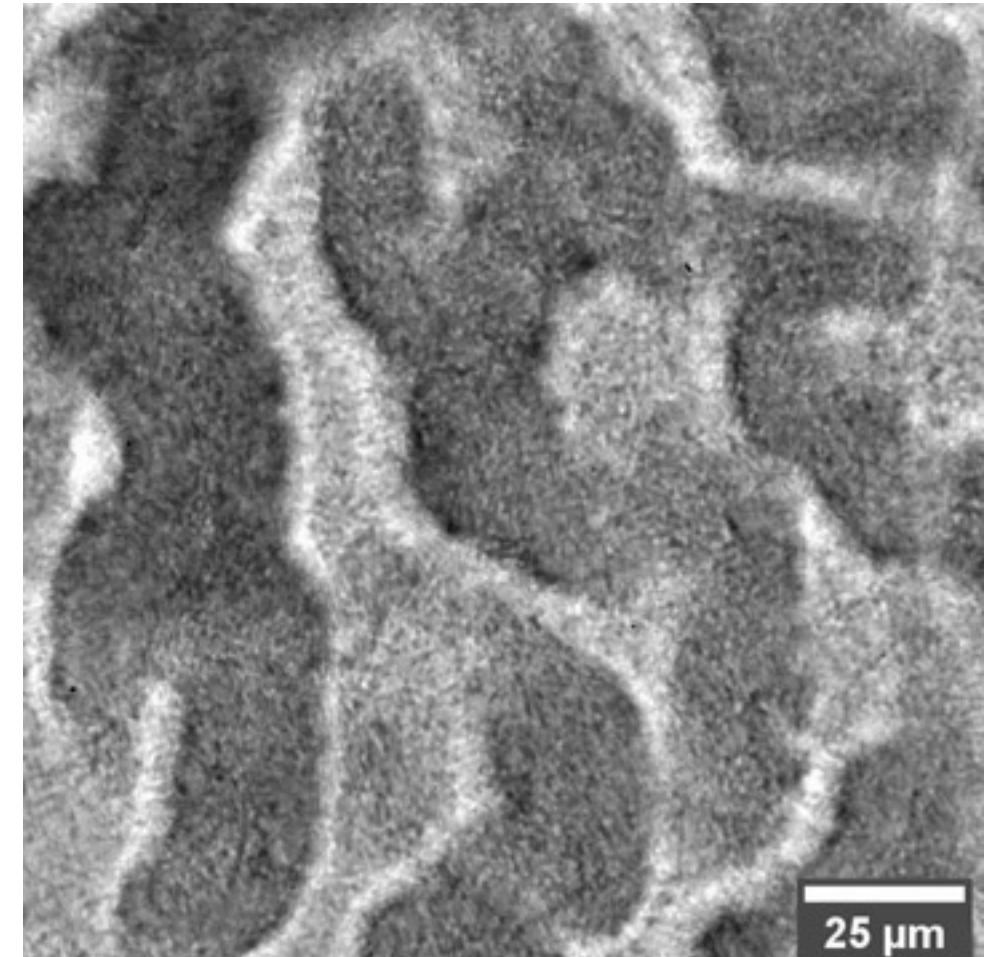
- similar to  $\Delta hag$ ,  $\Delta tasA$  could not engulf either paper or cellulose acetate discs
- The extracellular matrix (ECM) protein TasA was required for the expression of flagellar genes. Transcriptomic analysis revealed that TasA stimulated the expression of a specific subset of genes whose products promote motility and repress ECM production.

# Where are the location of motile cells ?

- Hollow channels are populated by motile bacteria.
  - A comparison between the activity map and the biofilm microstructure, as shown in the bright-field micrographs, reveals that the active areas are exclusively found inside the channels of the biofilm.
- Could explain spatial pattern of motile cell marker gene

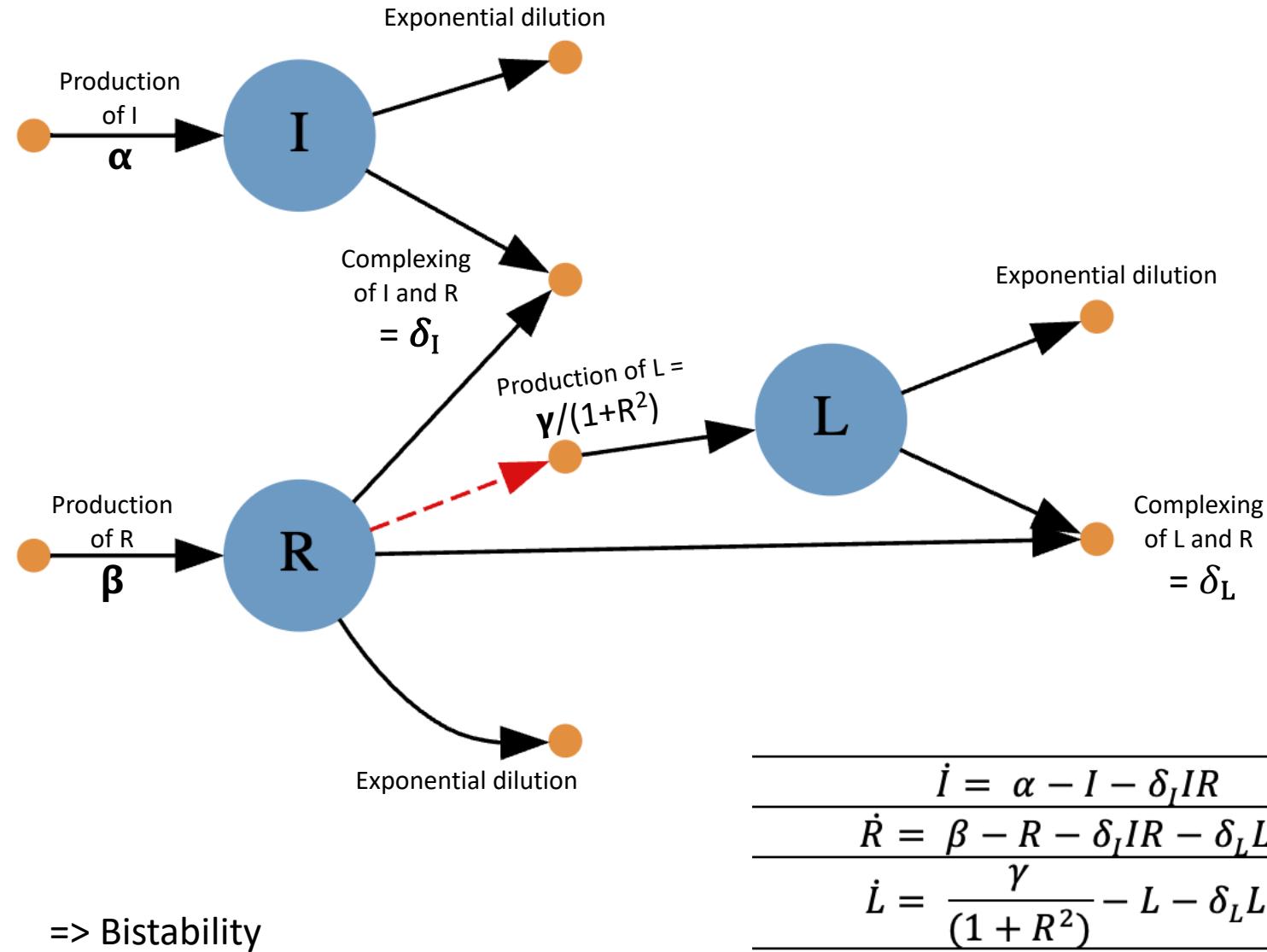


<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9208754/>



*P. aeruginosa*

# Current Model



$$\dot{I} = \alpha - I - \delta_I IR \quad (1)$$

$$\dot{R} = \beta - R - \delta_I IR - \delta_L LR \quad (2)$$

$$\dot{L} = \frac{\gamma}{(1 + R^2)} - L - \delta_L LR \quad (3)$$

Details of ComX: we know that ComX can activate the production of Surfactin (which activate KinC), but does it really increase matrix genes?

<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7463575/>

H: no ComX => less biofilm and matrix genes

Compare biofilms of WT and  $\Delta comQ$  (ComX deficient mutant)

- mostly on PS 216, a bit on NCIB 3610 (they are genetically quite similar)

	WT	ComX deficient mutant
Pellicle thickness, in MSgg medium at 16 h => Not support H	Thinner (12 $\mu\text{m}$ ) (but at 40h they looked the same)	formed a prominent pellicle biofilm, which appeared thicker (30 $\mu\text{m}$ )
Surfactin production at 40 h => support H	higher	Indeed, 8 times lower
=> So something missing here: lower Surfactin does not mean thinner biofilm		
- Something else replace surfactin in the mutant		
- Surfactin is not necessary for biofilm formation		
Hydrophobicity at 16h and 24h (not so diff at 40h) <a href="https://www.youtube.com/watch?v=1FRfrhzIPKY">https://www.youtube.com/watch?v=1FRfrhzIPKY</a>	less	Spread droplet more slowly => more hydrophobic If mean more BslA => not support H

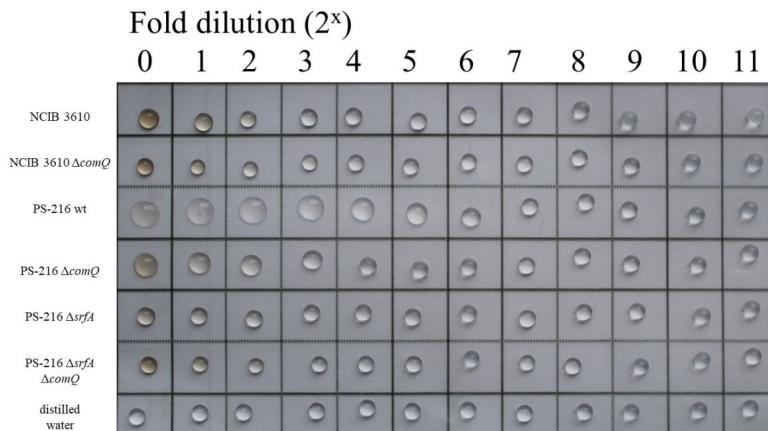
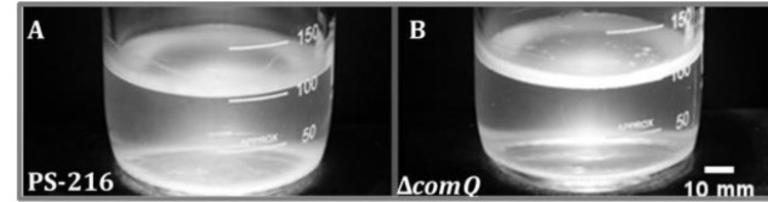
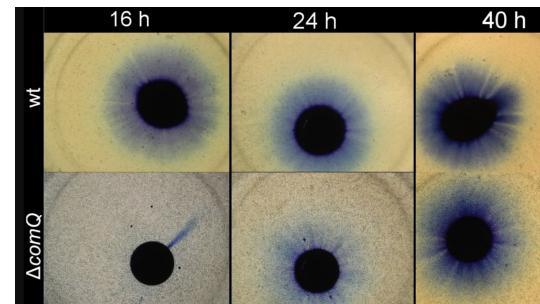


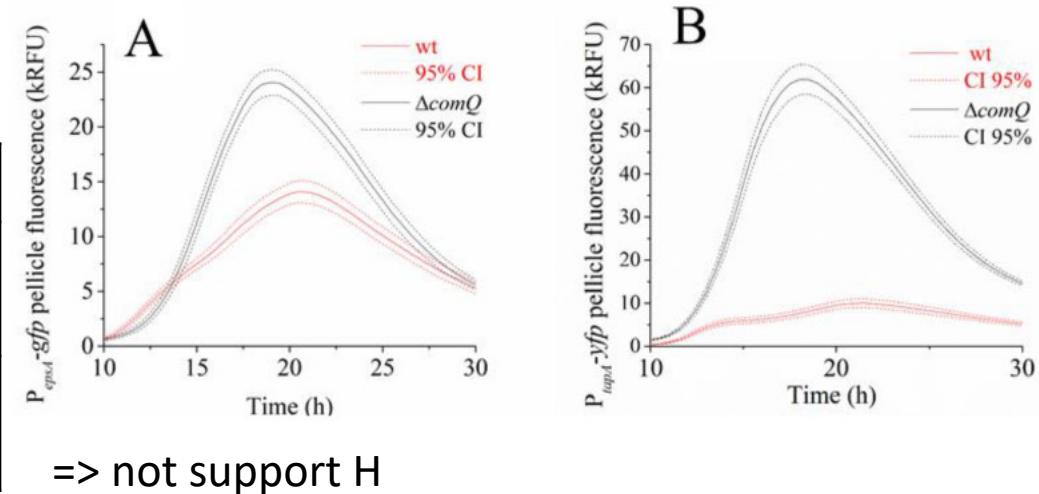
Figure S1: Semi quantification of surfactant concentrations in the pellicle biofilm spent media after 40 h of static growth in MSgg medium at 37 °C using a droplet surface wetting assay



# (Cont.) Does comX really increase matrix gene expression?

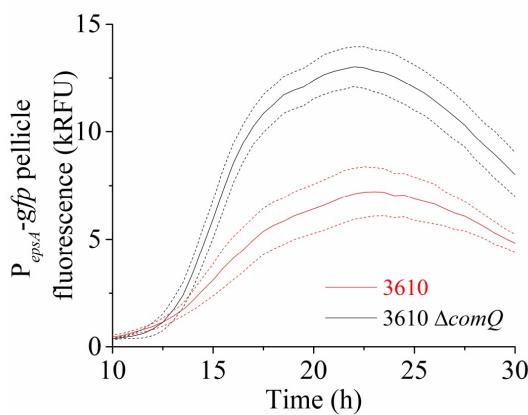
Though thicker pellicle, we should know the gene expression level

PS216	WT	ComX deficient mutant
activity of the $P_{epsA}$ at the population level	lower	a higher pellicle fluorescence intensity
$P_{tapA}$ at the population level	Always lower	a higher pellicle fluorescence intensity



Similarly, NCIB – same trends

– just less surfactin and less activity of  $P_{epsA}$



=> They kept looking at PS216

They wanted more details: spatiotemporal distribution of matrix producing cells

3 reporters:

- PepsA-gfp or PtapA-yfp reporters as markers
- a P43-mKate2 fluorescence reporter for metabolically active cells

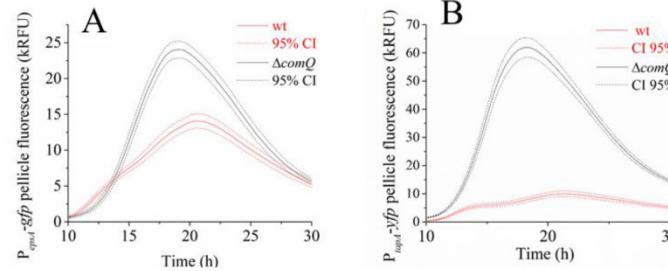
“After removing the liquid media from the well at indicated time points, we directly visualized the pellicles by confocal microscopy”

“We used the 100x immersion objective (NA 0.4) and roughly determined the bottom of the pellicle to set up a Z-stack”

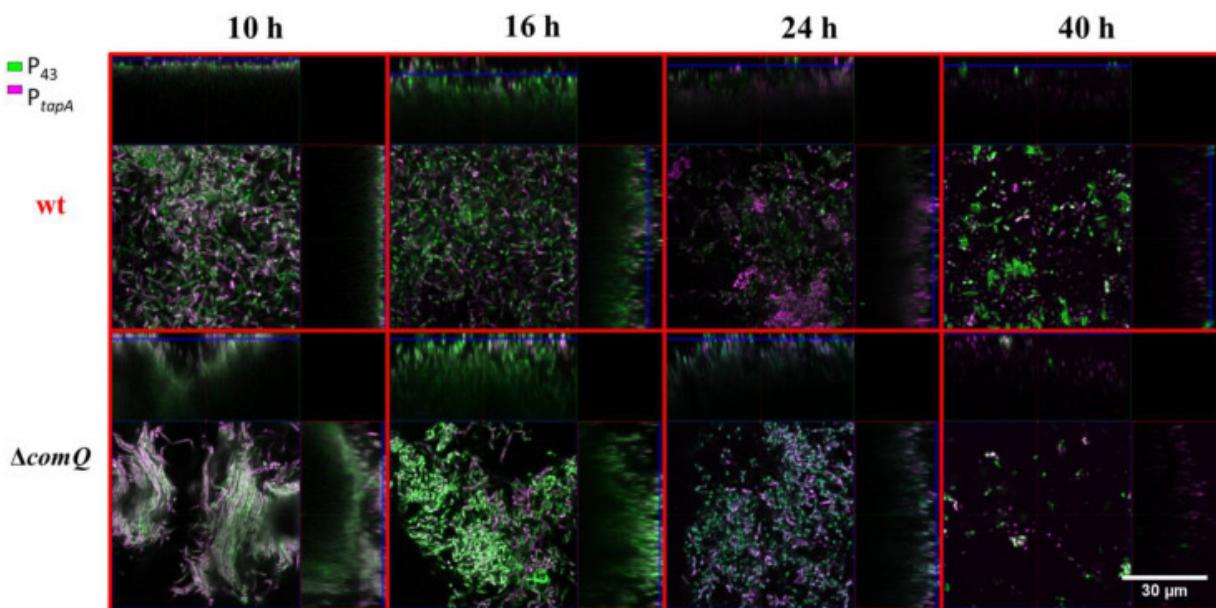
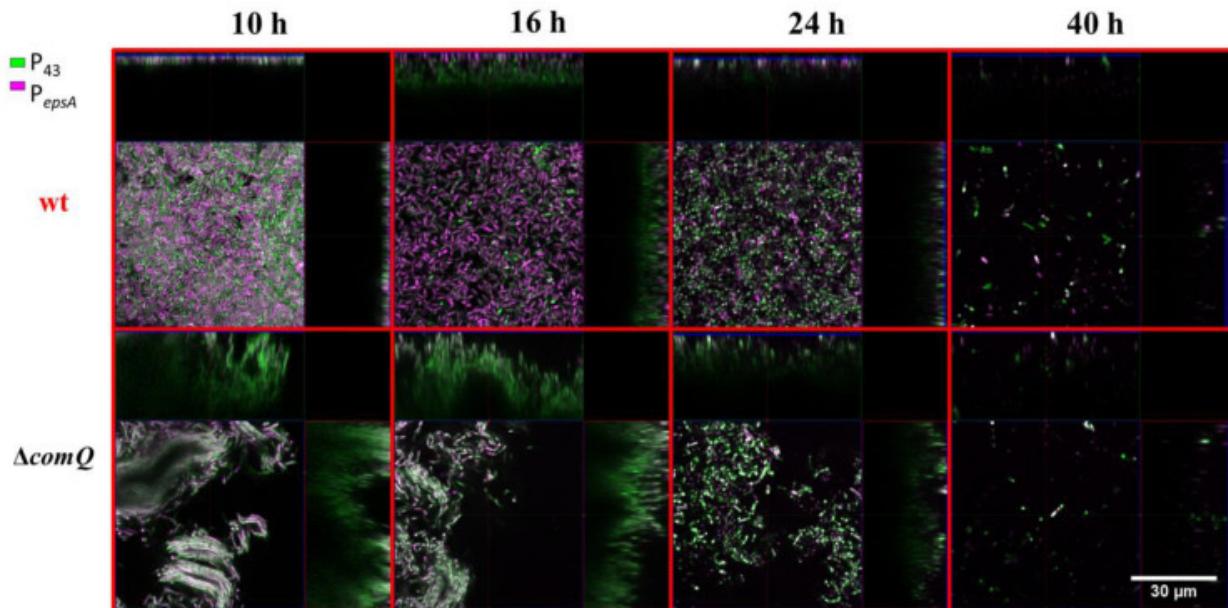
# (Cont.) Does comX really increase matrix gene expression?

Bottom of biofilm	WT	ComX deficient mutant
Cell density at 10 h	densely packed and evenly distributed	less densely packed
Cell morphology at the 10 h	No chaining	bundles of long chains of cells
proportion of cells with the active $P_{epsA}$ at 10 and 16h	Higher proportion = $P_{epsA}$ activated promoter cells occupied the bottom of the pellicle	=> Support H  No substantial difference in the activity of the $P_{epsA}$ at late time points and of PtapA promoter between the two strains at any time points => not support H

=> ComX def mutant show hallmarks of the biofilm phenotype first => not support H



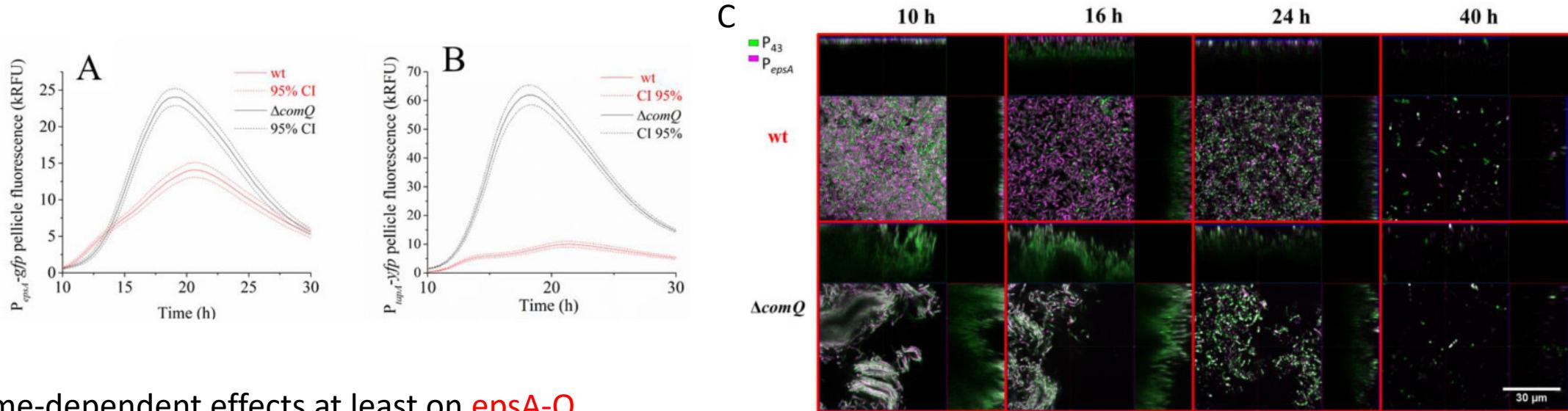
By 40 h, we detected only a few P43 fluorescing cells in both strains (biofilms were still visible by the naked eye and look similar in both strains)



# (Cont.) Does comX really increase matrix gene expression?

Now what we know so far

- Although surfactin production in comX mutant (at 40h) was indeed lower and the proportion of cell expressing epsA-O at the bottom of the biofilm was also lower in comX mutant at 10h and 16h, much evidence did not support H:
- WT had a thinner and less hydrophobic biofilm at early timepoints (~16h), the bulk promoter activity of matrix genes was lower in WT, the mutants started chaining first, and no difference in the proportion of cell that expressed tapA at any time points and epsA-O at late timepoints.



Time-dependent effects at least on **epsA-O**.

During early stage, ComX does something to increase **epsA-O** expression at cellular level (figC) (they did not quantify surfactin level at early points, so we cannot tell if it is through surfactin or not).

During late timepoints, ComX seems to have the insignificant effect and even a negative effect on at **espA-O** population level (figA) => this suggests the importance of cell density when quantifying gene expression

# (Cont.) Does comX really increase matrix gene expression?

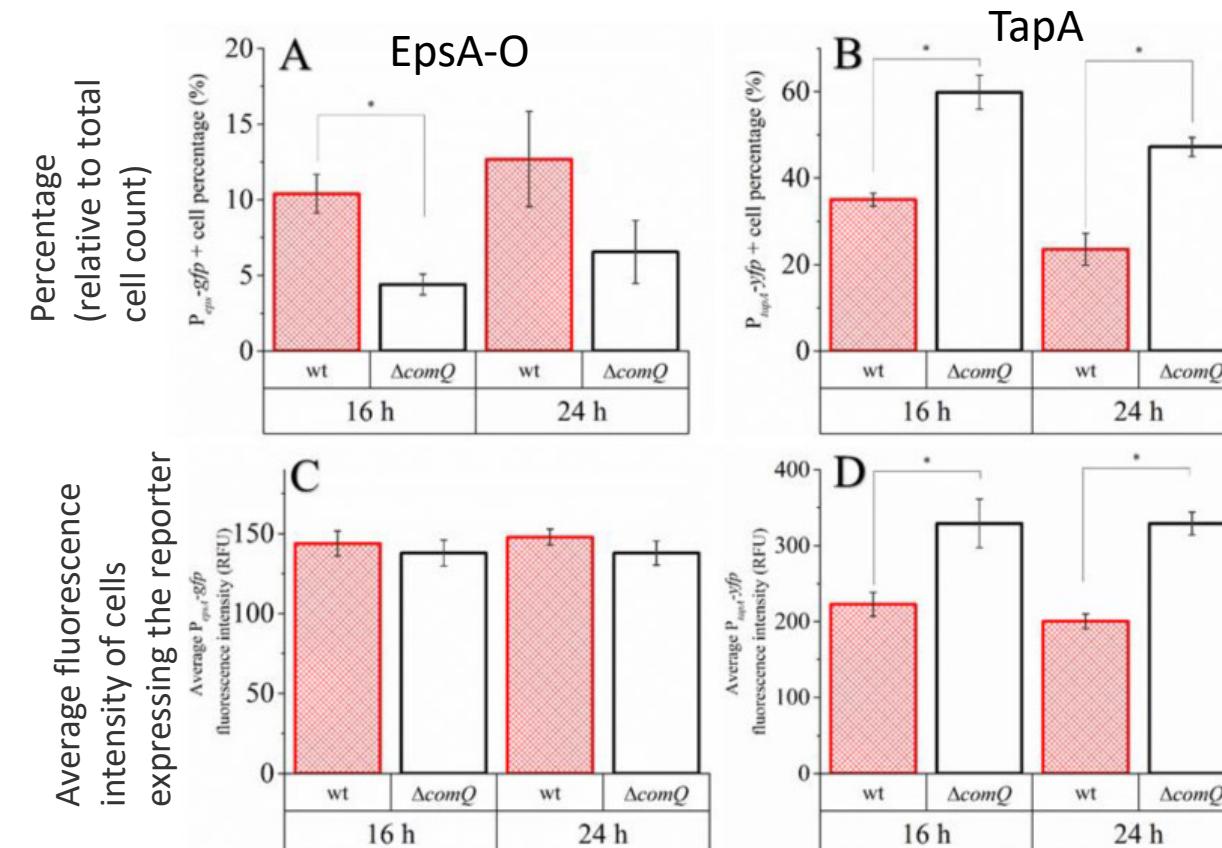
"the microscopy of the undisturbed pellicle only provided qualitative results of the distribution of cell types" => flow cytometry

"disrupted the pellicle at 16 and 24 h time points and employed flow cytometry on the cell suspension to determine the percentage of cells that express the *epsAO* and *tapA* operons."

- =>
- 2 measurements
    - the relative percentage of cells expressing each operon
    - the level of transcription at a single cell level.

ComQX system increases the fraction of cells expressing the *epsA* operon during **early** biofilm development without changing the average intensity of PepsA transcription.

=> only support H for early time points



ComX decreases the fraction of cells with PtapA activity and the average activity in those cells

=> Not support H

# (Cont.) Does comX really increase matrix gene expression?

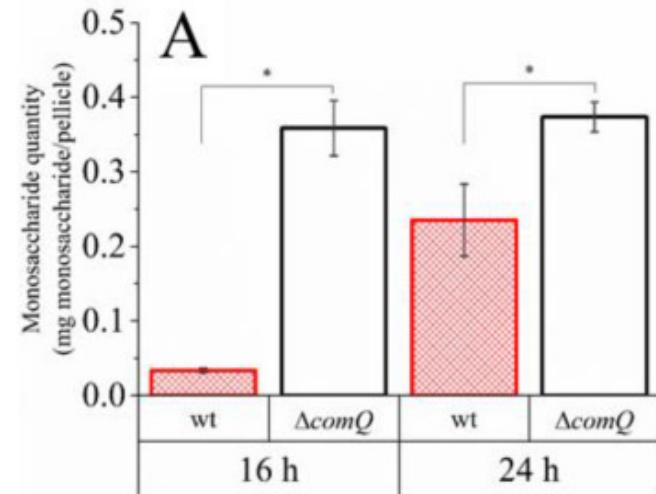
"essential to verify whether ComX deficient mutants preserve this pattern at the level of production of matrix components"

extracted the extracellular polymers

Quantified by reducing sugar assay and Bradford assay

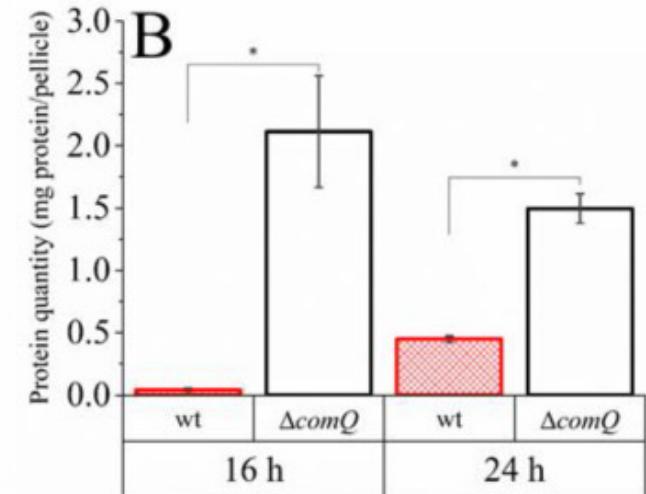
$\Delta comQ$  biofilms harbored a higher amount of monosaccharide per pellicle than WT! (though they have lower proportion of cells expressing *epsAO* operon) at all time points

Expression ==> translate to level of component?



Not expected

- ⇒ Both not support H
- ⇒ Agree with bulk promoter activity

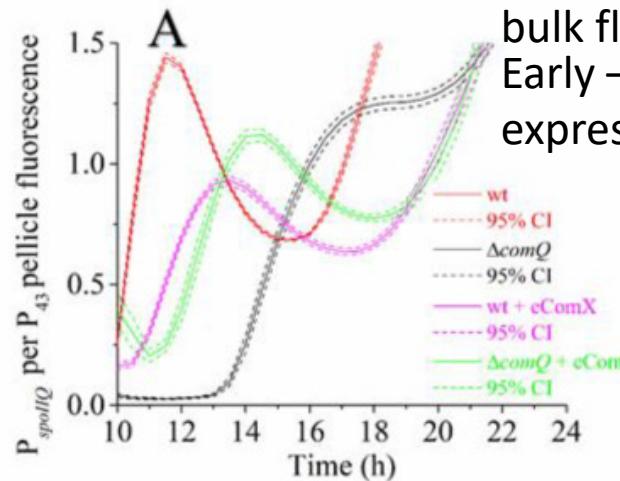


As expected

"At this point, we cannot explain the molecular basis of this observation, but only speculate that the synthesis of matrix polysaccharides might be regulated by additional mechanisms that are under the influence of ComX and are independent of PepsA promoter activity."

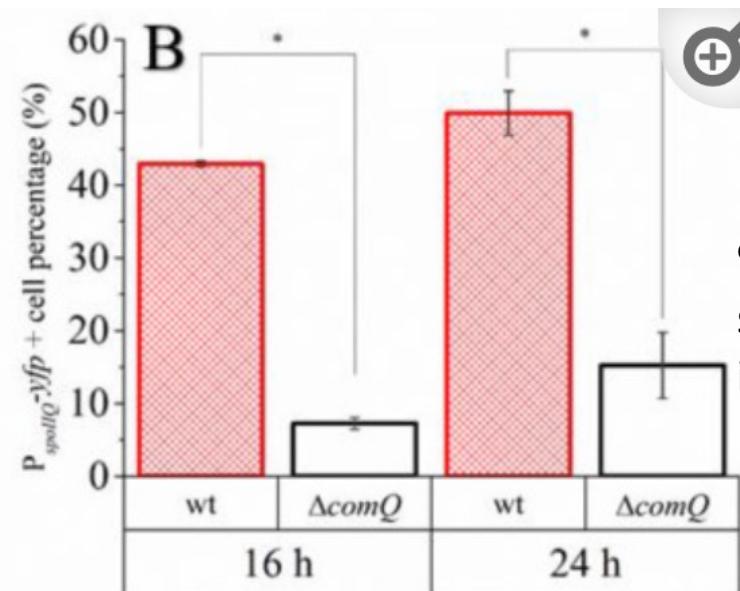
# (Cont.) Does comX really increase matrix gene expression?

We may need to look at ComX effect on sporulation. If ComX level influences matrix gene expression through Spo0A-P level, it should then affect sporulating gene e.g. *spoIIQ*



bulk fluorescence by fluorometry  
Early – **WT** has a higher spore gene bulk expression than mutant

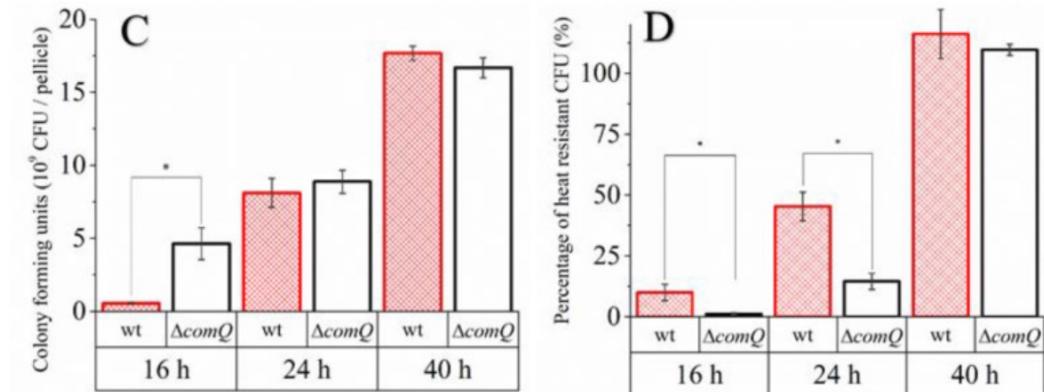
WT+eComX has similar pattern with **mutant+eComX**  
eComX change **WT** expression



Flow cyto  
% of cells expressing spore genes is higher in WT

=> WT enters sporulation after only 10 h of incubation.

Total pellicle biofilm CFU counts & %heat resistant CFU per total pellicle CFU



**Mutant** has a significantly higher CFU count in the pellicle at the 16 h

At 16 h, the **WT** pellicle therefore contained 5-fold and at 24 h, 3-fold more heat resistant CFUs

After 40 h, all the pellicle CFUs were heat resistant in both strains