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Bachelor Thesis

The molecular mechanism of surfactin immunity in *B. subtilis*

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Abstract

The differentiation of cells into biofilms is a crucial step in the survival of a bacterial colony. In the gram-positive bacterium *Bacillus subtilis* this differentiation is interesting, as the behaviour of cells in communities might enlighten ways that bacteria behave like multicellular organisms. One of the differentiation pathways in *B. subtilis* is differentiation into matrix producing cells. ComX plays a major role in the molecular mechanism of the initiation of matrix production. ComX initiates surfactin production, which in turn inhibits the blockage of transcription of matrix related genes, activating the matrix production. Interestingly, recent research shows that surfactin producing cells are unable to respond to surfactin and become matrix producers. In other words: they become immune to surfactin. In this review I discuss a model of the molecular mechanism behind this surfactin immunity that was proposed by Lopez et al. (2009). I discuss several papers to validate this model, which lead to an improved model of surfactin immunity. Additionally, I describe how these papers clarify the interaction between Spo0A, MecA, ClpCP and gene transcription of *sinI* in the last step of the molecular mechanism of surfactin immunity.

Contents

Introduction

An important property of cells of a multicellular organism is the ability to communicate with other cells. To accommodate this, a large range of signalling molecules are excreted and receptors for these molecules are expressed.

Bacterial cells are not part of a multicellular organism, but they do exhibit ways of intercellular communication, which leads them to behave in a multicellular manner (Aguilar et al., 2007).

In *Bacillus subtilis,* intercellular communication (generally) relies on gradient-dependent responses, meaning that several compounds are continuously excreted. Once a threshold is met, cells are able to respond to the signal. One of the compounds that act in this way is the polypeptide ComX, a so called self-sensing (or quorum-sensing) protein (Bareia et al., 2018).

ComX is constantly expressed, modified and secreted by every cell in a colony of *B. subtilis.* This creates an extracellular gradient that is representative for the amount of cells in the environment (Bareia et al., 2018). Cells in the environment can respond to ComX by initiating matrix production, one of various cell fates (Shank & Kolter, 2011). ComX has interactions with ComP, that causes this membrane protein to phosphorylate ComA. The activated transcription factor ComA-P will activate transcription of the *srf* gene, which codes for the polypeptide Surfactin (figure 1). (Okada et al., 2017)(Lopez et al., 2009). Surfactin is then secreted into the extracellular environment.

In this environment, cells are able to interact with surfactin through KinC. This will initiate a signalling cascade, in which a transcription factor Spo0A is phosphorylated, activates the transcription of the inhibitor SinI. SinI will activate transcription of matrix-associated genes, by releasing the transcriptional inhibitor SinR (figure 1)(Lopez et al., 2009).

Interestingly, Lopez et al. (2009) reported that surfactin producing cells become immune to surfactin. This initiates a sort of biological timing in the development of the biofilm, or in other words, a unidirectional interaction leading to a signalling cascade of cells that should or should not differentiate to a certain sort of cell (Alberts et al., 2015).

Regulation of cell differentiation, as well as a heterogeneous differentiation in a bacterial community is important to find the optimal state of fitness (Veening et al., 2008). Timing plays an important role in the distribution of these differentiations.

In order to fully understand this extracellular communication and timing mechanism, it is important to ascertain the molecular mechanism behind the earlier described surfactin immunity of surfactin producing cells.

Here, I discuss how unidirectional communication in *B. subtilis* was discovered and describe the molecular mechanism of surfactin immunity as proposed by Lopez et al. (2009). Additionally, I aimed to confirm the model by discussing two publications and I propose an improved model of the molecular mechanism of surfactin immunity in surfactin producing cells.

Surfactin immunity

The unidirectional communication in *B. subtilis* was found by Lopez et al. (2009), during experiments to clarify the molecular mechanism of matrix production in colonial cells. Experiments to monitor how many cells were matrix producers and which promoters were involved, were designed based on the information in figure 1.

The authors designed three transcriptional fusions in order to be able to monitor the cells. The promoters: P_{comQXP}-*yfp* (Yellow Fluorescent Protein (YFP) expressed when ComX promoter is activated), P_{srfAA}-*yfp* (YFP expressed when surfactin promoter is activated) and P_{yqxM}-*cfp* (Cyan Fluorescent Protein (CFP) expressed when matrix production is activated) were separately expressed in a strain. To make sure that the population harboured various types of cells

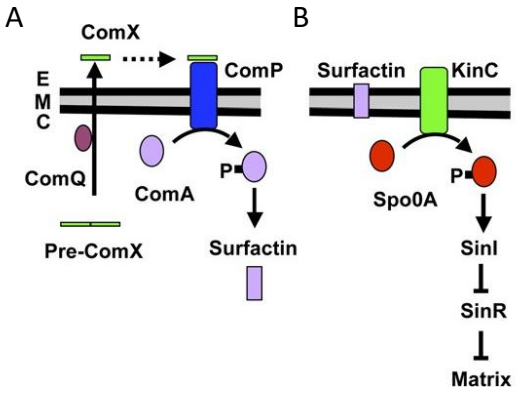

Figure 1. Schematic representation of the regulatory mechanism that controls matrix production in *B. subtilis* (Lopez et al.,2009). **A.** Activation of the *srf* gene that codes for surfactin. *B. subtilis* cells continuously transcribe *comX*, which codes for a pre-ComX polypeptide. ComQ executes posttranslational modifications to this pre-ComX and transports it over the cell wall, releasing it in the extracellular environment. Once ComX reaches a certain threshold, it will activate ComP to phosphorylate ComA. Next, ComA-P, a transcriptional regulator, will activate transcription of *srf.* **B.** The molecular mechanism of the cellular response to surfactin. Surfactin has interactions with KinC, allowing it to phosphorylate Spo0A. Spo0A-P will activate transcription of SinI. SinI blocks the transcriptional inhibitor SinR, eventually activating (or de-repressing) matrix production (Lopez et al. 2009).

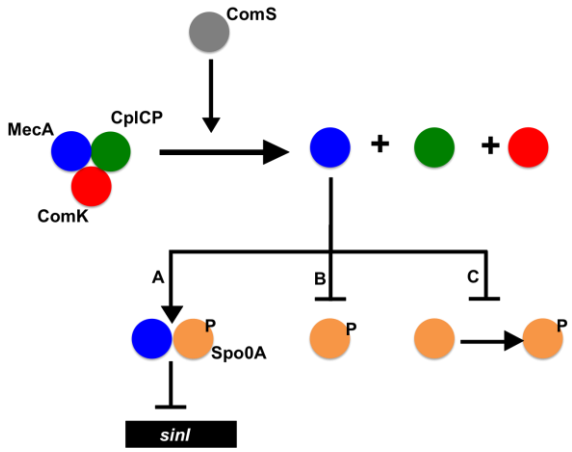

Figure 2. Schematic representation of the molecular mechanism of surfactin immunity in surfactin producing *B. subtilis* as proposed by Lopez et al. (2009). ComS initiates the dissociation of the ComK-ClpC/P – MecA complex either by dissociating all three components or by only dissociating ComK from the ClpCP-MecA complex (not shown). Lopez et al. propose that **A.** Spo0A and MecA interaction that inhibits *sinI* transcription. It may also be that **B.** inactivation of Spo0A-P by MecA, or **C.** Inhibiting the phosphorylation of Spo0A, leads to the suppression of matrix production. (Prepiak et al., 2011)(Tanner et al., 2018)

these strains were incubated to the phase where biofilm production was well on its way. The authors were able to use flow cytometry to count the cells expressing the promoters, because the cells that express certain promoters were fluorescent.

The expression of P_{comQXP}-*yfp* was unimodal, which indicates that either all or no cells were expressing the promoter throughout the biofilm population. Interestingly, P_{srfAA}-*yfp* showed a bimodal expression, which indicates that part of the population does and another part of the population does not express this promoter. This meant that a part of this biofilm population was not responding to ComX by producing surfactin. The authors later explain that this is due to matrix production, because this blocks the activity of ComP, blocking surfactin transcription (figure 1).

Last, the P_{yqxM}-*cfp* promoter was expressed bimodal as well and expression of the P_{yqxM}-*cfp* promoter was found in about 20% more cells than the amount of cells expressing P_{srfAA}-*yfp.* This shows that the response of cells to produce surfactin or was not occurring in all cells.

One explanation for this observation was that these processes were occurring in different cells. To confirm this idea Lopez et al. (2009) performed another flow cytometry experiment as well as a microscopy experiment, using a strain that contained both P_{srfAA}-*yfp* and P_{yqxM}-*cfp.* Data in both experiments showed no overlap in the fluorescent signal.

This confirms that the surfactin promoter and the matrix-producing promoter were not active in the same cells. In other words, surfactin producing cells are unable to respond to surfactin and therefore are 'immune' to surfactin.

This introduces a unidirectional regulation of cell differentiation, which is important for biofilm development. Differentiation pathways are energy-intensive pathways and avoiding excessive transitions between states will therefore cause for a more efficient use of energy in the cell. Additionally, heterogeneous populations appear to have a higher fitness compared to homogeneous populations, making it important for the biofilm to have various cells available to ensure survival in various conditions (Prepiak et al., (2011)) (Veening et al. (2008)).

A model for the molecular mechanism of surfactin immunity

Lopez et al. (2009) propose a molecular mechanism that might explain the surfactin immunity in these surfactin producing cells.

Their model, as described in figure 2, starts with ComS, which is simultaneously transcribed with *srf.* The protein ComS can dissociate MecA and ClpCP from ComK (Hamoen et al., 1995). ComK is the key protein to induce the so-called K-state of *B. subtilis*, during which the cells are able to take up DNA from the environment (Hahn et al., 1996).

MecA and ClpC are known to target proteins for degradation. MecA consists of a C-terminal domain (CTD) and N- terminal domain (NTD), forming a dimer with several sites that can interact with other proteins. The CTD can interact with ClpC, whereas the N-terminal domain interacts with ComS and ComK. Because they compete for this binding site, this confirms the first statement that ComS releases ComK from MecA (Persuh et al., 1999).

The release of ComK from MecA allows ComK to accumulate in the cell. As Lopez et al. (2009) argue, this accumulation is regulated by an intrinsic system, which we will not discuss here. We can state however that this system does not affect matrix production and is therefore not of our interest.

The increase of ComK (with or without ClpC bound) may, however, lead to an accumulation of MecA. I hypothesize that this may result in three options, (figure 2A,B and C respectively) where either the activity of Spo0A-P or the phosphorylation of Spo0A are inhibited, or Spo0A and MecA bind an inhibit *sinI.*

MecA blocks energy-intensive pathways

To clarify the key role of MecA in this process, Prepiak et al. (2011) present data of experiments on a population-level. They used a strain that over expressed *mecA* (for simplicity we refer to this strain as 'the *mecA*$^+$ strain') and a strain that had a *mecA*

deletion ('the Δ*mecA* strain') to show that MecA influences gene expression. Interestingly, the colony's physiology was altered when these alterations were expressed. The *mecA*$^{+}$ colony appeared opaque, resembling colonies in which the sporulation pathway has been blocked. The *ΔmecA* colony had a rough surface, which is unnatural to *B. subtilis* colonies. An additional experiment showed that the phenotype of the *ΔmecA* strain can be rescued by expressing *mecA*$^{+}$.

Additionally, using a luciferase reporter system of their own design they were able to follow gene expression in real time. Prepiak et al. (2011) showed that *mecA*$^{+}$ prevents expression of *spoIIG* and that *ΔmecA* reduces the amount of cells that express *eps. SpoIIG* is a gene expressed during sporulation, whereas *eps* encodes several signalling compounds used in cellular communication.

The *ΔmecA* strain showed a reduction of *eps* expression which was not uniform in the population. To confirm this observation, the authors created two transcriptional fusions: *eps-cfp* and *spoIIE-gfp. Eps-cfp* expressed in *ΔmecA* showed that indeed, some cells did not express *eps.* In contrast, expression of *spoIIE-gfp* in the *mecA*$^{+}$ lead to the absence of any sporulating cells in the colony.

This indicates that MecA blocks transcription of these two genes, and possibly more for the mechanism behind this inhibition is not explained at this point. It also shows that MecA blocks genes that are known to initiate energy intensive differentiation pathways (sporulation for *spoIIE*), indicating that MecA plays a role in the regulation of biofilm formation. As mentioned in the introduction, this can have a positive effect on the fitness of the bacterial colony.

Additionally, the gene *spoIIE* are known to be regulated by Spo0A (Statola et al., 1992). Therefore effect of MecA on the expression of these genes indicates an interaction between MecA and Spo0A.

MecA interacts with ClpCP to block transcription

MecA may not be the only factor involved in the molecular mechanism of surfactin immunity, for we have not discussed the possibility of ClpCP playing a role in the mechanism. ClpCP may play a role in the mechanism, because it is part of the ClpCP-MecA-ComK complex before ComS releases ComK.

When bound to MecA, ClpCP is known to target proteins for degradation. It could be that the release of ComK allows the complex to target Spo0A-P for degradation, inhibiting transcription activation of *sinI*.

To see whether ClpCP effects transcription, Prepiak et al. (2011) followed *eps* expression using a *eps-LacZ* fusion and a *ΔclpC* mutation in the *mecA*$^{+}$ and Δ*mecA* strain. Once a *ΔclpC* mutation is introduced to the *mecA*$^{+}$ strain, *eps* expression exceeds wild type expression of *eps.* This suggests that ClpC and MecA are both able to suppress transcription of genes associated with Spo0A.

Spo0A phosphorylation is needed for inhibition of transcription

One of the possible ways that MecA and ClpC inhibit transcription may be that they bind Spo0A and inhibit its phosphorylation, because Spo0A needs to be phosphorylated to be able to regulate transcription.

Prepiak et al. (2011) used a *sad67* mutation that makes the activity of Spo0A independent of phosphorylation and removes 19-aminoacid residues from its receiver domain. In the *mecA*$^{+}$ strain, in combination with the *spoIIE-lacZ* fusion to follow its expression, the authors added the *sad67* mutation (under a P_{spac} promoter, making *sad67* inducible with IPTG). Prepiak et al. concluded that the *sad67* mutation bypasses the repressing effect of the *mecA*$^{+}$ strain. This indicates that, in order to inhibit transcription, MecA needs to interact with or inhibit the phosphorylation of Spo0A. It could also be that the deleted region of 19 amino acids was inhibiting the proteins to bind.

Spo0A interacts with MecA*in vitro*

A second option is that MecA and ClpC target Spo0A for degradation. To be able to state this, it is important to show evidence that supports the statement that Spo0A and MecA interact. Prepiak et al. performed *in vitro* experiments that confirm that these proteins interact.

Using surface plasmon resonance (SPR) experiments with histagged and non-histagged MecA bound to the chip they were able to show that Spo0A and MecA interact in a dose-dependent way. They flooded the MecA-chip with Spo0A and turned the experiment around as well, flooding a Spo0A-bound chip with MecA. The interaction resembled that of Spo0A and Spo0A-anti-bodies.

Additionally, to determine where the interaction of Spo0A occurs on MecA, the two domains of MecA were separated and tested in additional SPR experiments. Interestingly, it appears that Spo0A interacts with both of the domains, in contrast to the other interactions of MecA, that are domain specific.

Importantly, this shows that ComK and Spo0A bind to MecA in a different way and therefore do not antagonize or compete for the same binding area on the protein. This lack of competition was confirmed by an SPR experiment where the authors put MecA on the chip and flooded it with both Spo0A and the part of ComK that binds to MecA. Both compounds bound

simultaneously, which may indicate that MecA does not target Spo0A for degradation the same way it targets ComK.

Additionally, the NTD and CTD alone were not sufficient for the inhibition of gene transcription. Therefore, the whole of MecA is needed to interact with Spo0A-P and inhibit transcription (Prepiak et al., 2011).

This shows that Spo0A does interact with MecA and confirms the *sad67* mutation experiments that Spo0A does bind and that, therefore, the phosphorylation of Spo0A plays an important role in the interaction.

Spo0A is not targeted for degradation by MecA

The difference between the binding site for ComK and Spo0A indicates that perhaps, Spo0A is not targeted for degradation and that this is not the way of matrix inhibition in the surfactin immunity pathway.

Indeed, Prepiak et al. (2011) show that MecA does not target Spo0A for degradation. In Western blot experiments they followed Spo0A and MecA expression in *mecA*$^{+}$, Δ*mecA* and wild type. Interestingly, they observe slight increase in Spo0A expression in the Δm*ecA* strain. They suggest that this effect is probably due to the positive feedback loop where Spo0A-P can initiate its own expression.

To confirm that MecA does not target Spo0A for degradation the authors used an *in vitro* degradation assay which showed, in contrast to the active ComK degradation in the experiment setup, no degradation of Spo0A.

Additionally, they show that MecA does not decrease the stability of Spo0A. They isolated the proteins and tested them for degradation in a degradation assay. By adding puromycin protein production was halted (Pestka et al., 1971) and little decay was observed in both strains.

Two models for the interaction of MecA, CplCP and Spo0A-P

Now that we know that MecA inhibits transcription through interaction with Spo0A-P, does not degrade it and does not limit its activity, only one option remains: inhibition of the transcription of *sinI*. Therefore, it is important to decide on the mechanism that describes this inhibition. Prepiak et al. (2011) proposed two mechanisms for this transcriptional blockage, as can be found in figure 3. The first mechanism (upper left of figure 3) states that after release from ComK, MecA is free to bind to Spo0A and does this when Spo0A bound to the promoter site (on-target binding). The second mechanism describes MecA preventing Spo0A from binding to the promoter (off-target binding).

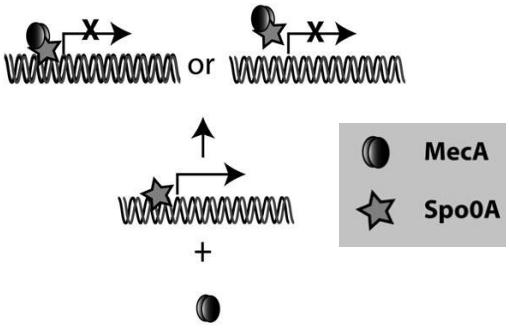

Figure 3. A schematic representation of the two models of transcriptional inhibition by the Spo0A-MecA complex as proposed by Prepiak et al. (2011). Either MecA interacts with Spo0A after binding to the promoter site (upper left), or MecA prevents Spo0A from interacting with the promoter site (upper right).

MecA inhibits transcription on-target

Discerning the difference between off- and on- target binding is challenging, for addition of MecA would repress expression and deletion of MecA would increase expression in both mechanisms.

However, the difference can be observed when looking at genes that are negatively regulated by Spo0A: off-target binding to MecA would in this case greatly increase expression of the according gene in an over expressing strain (Tanneret al., 2018).

To test if this was happening in this mechanism, Tanner et al. (2018) created transcriptional fusions of promoters known to be positively or negatively regulated by Spo0A. The fusion used luciferase, similar to what was done by Prepiak et al. (2011). The positively regulated promoters were P_{spoIIG}, P_{spoIIE} and P_{spoIIA} and the negatively regulated promoters were P_{araB}, P_{med} and P_{yuxH}. The positively regulated promoters were combined with *Δspo0A* to control for the regulation. Indeed, these promoters were very little expressed. Additionally, the activity of the negatively regulated promoters with *Δspo0A* had notably increased expression, confirming the constructs and the regulation Spo0A on these promoters (Tanner et al., 2018).

Next, the authors expressed the six constructs in strains containing alike done by Prepiak et al.(2011): *mecA*$^{+}$, Δ*mecA* and wild type. Tanner et al. (2018) observed an increase in the expression of the positively regulated promoters in the *mecA* deletion strain, compared to the wild type. This effect was not visible in the negatively regulated strains. Therefore the off-target mechanism is not applicable to this system, but an interaction between the inhibitory complex and the promoter is required.

The earlier described interaction of MecA with the phosphorylation of Spo0A can therefore be explained by the fact that Spo0A only binds as Spo0A-

P to the promoter and that MecA binds on-target. This rejects the mechanism of off-target binding, which supports the fact that MecA does not target Spo0A for degradation, for this would most likely happen off-target.

MecA affects SinI and SinR

I therefore propose that the effect of MecA on Spo0A-Ps ability to activate *sinI* transcription is responsible for the blockage of matrix production. Prepiak et al. (2011) showed that the blockage of the *eps* pathway by MecA is mainly done via SinI and SinR, which confirms this proposition.

The authors visualised the expression of SinI in the *mecA*$^{+}$ and the *ΔmecA* strain in a Western blot experiment. In *mecA*$^{+}$ the concentration of SinI was decreased and in the *ΔmecA* strain, SinI was increased to levels higher than found in the wild type. Additionally, the *sinI-LacZ* fusion showed a similar response. This meant that MecA down regulates the expression of *sinI-lacZ*.

Because *sinI* is positively regulated by Spo0A-P, the on-target binding of MecA to Spo0A blocks transcription of *sinI* which blocks *eps* transcription, but could lead to the inhibiting activity of SinR to stay intact, suppressing matrix production and creating the surfactin immunity.

ClpC and MecA interact to inhibit Spo0A

Finally, because ClpCP has an influence on *eps* gene expression in the same way that MecA does, ClpC might be needed for the interaction with Spo0A-P.

Tanner et al. (2018), presented data that confirm this hypothesis in an *in vivo* experiment using the six luciferase fusions from before to follow transcription regulation. They expressed these constructs in *mecA*$^{+}$ and added a *ΔclpC* mutation. If indeed ClpCP was needed for the interaction, then this construction would show an increase in expression compared to when the *ΔclpC* mutation was not added.

Interestingly, the authors were able to present data that confirms this hypothesis, showing that ClpC is important in the mechanism. Additionally, the *ΔclpC* mutation initiated an earlier onset of gene expression than the wild type.

Besides this *in vivo* experiment, *in vitro* experiments also confirm that ClpC and MecA interact to inhibit transcription. Tanner et al. (2018) used the P_{spoIIG}- *luc* to follow gene expression and added Spo0A-P. As expected, this activated gene expression. Addition of MecA did not alter gene expression, which confirms that MecA cannot inhibit Spo0A off-target.

To test the effect of ClpC on expression *in vitro*, it was important to minimize any side effects by adding ATP to the reaction. Interestingly, addition of only ClpC decreased expression on its own. Adding combination of ClpC and MecA reduced expression even more, which indicates that these compounds need to interact in order to inhibit transcription.

Conclusion and Discussion

In this review I described how Lopez et al. (2009), identified that surfactin producing *B. subtilis* cells become immune to surfactin. This immunity plays a role in biofilm development and indirectly increases the fitness of the bacterium (Veening et al., 2008). The model of the molecular mechanism that describes this surfactin immunity, as Lopez et al. (2009) propose may involve ComS.

ComS is transcribed together with *srf*, the surfactin coding gene. The expression of ComS in the cell would release ComK from MecA, leaving this protein free for interaction with the transcription factor Spo0A-P. MecA would then inhibit the activity of Spo0A-P. This protein is required for the cells to respond to surfactin and blocking it would make Spo0A unable activate *sinI* transcription. The exact mechanism of this last step remained unclear, but it would block the activation of matrix production, creating an immunity to surfactin (figure 2).

To confirm this model and to clarify the last step, I discussed publications that present data on the interaction between Spo0A and MecA. First, I described how Prepiak et al. (2011) were able to show that MecA and Spo0A interact *in vivo*, using deletion and overexpression of MecA to show that an increase of MecA, decreases Spo0A associated gene expression. Additionally, *in vitro* SPR experiments showed that MecA binds to Spo0A. The authors also showed that MecA was not the only factor important for the transcriptional inhibition, for the ATPase ClpC also had an inhibitory effect on gene expression *in vivo*.

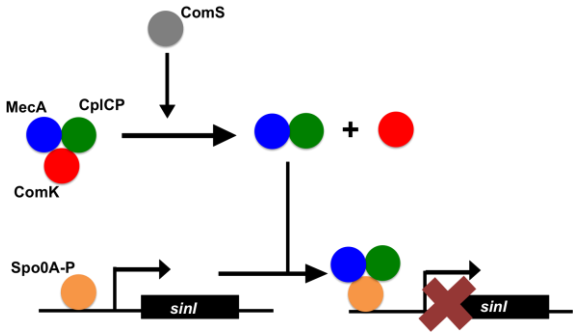

Figure 4. Schematic representation of the molecular mechanism of surfactin immunity in surfactin producing *B. subtilis* as described in this review. ComS initiates the dissociation of the ComK-ClpC/P – MecA complex either by dissociating all three components or by only dissociating ComK from the complex (not shown). This is followed by the binding of Spo0A-P to MecA and ClpCP, which inhibits transcription of *sinI* while Spo0A remains bound to the promoter. This in turn leaves SinR to inhibit transcription of matrix-associated genes, blocking the cellular response to surfactin.

Additionally, Tanner et al. (2018) show that MecA inhibits the activity of Spo0A-P on-target, as opposed to off-target, rejecting the idea that Spo0A is prevented from phosphorylation or limited in its activity to activate *sinI* transcription in this way.

The improved model

Because the interaction of Spo0A and MecA is dose dependent, I confirm that it is likely that the first step of the mechanism is the release of ComK from MecA-ClpC. Because ClpC is necessary, Tanner et al. (2018) conclude that the inhibitory complex exists of MecA, Spo0A and ClpC. Unfortunately, I was not able to discern whether or not ClpC is released from MecA after ComS interacts with MecA. I however reason that, because ComS and ComK specifically compete for the same binding site on MecA, it is unlikely that ClpC is released from MecA. Additionally, because MecA increases the activity of ClpC there is no apparent reason for these two proteins to release their interaction and therefore they would remain bound after ComK is released.

After release, concentrations MecA-ClpC and ComK increase. This will lead to the interaction of MecA-ClpC with Spo0A.

The final step in the model of surfactin immunity, as proposed by Lopez et al. (2009) was partially incorrect. MecA does not interact with Spo0A or its phosphorylation, but I argue that the on-target inhibition of transcription by the MecA-ClpC-Spo0A complex inhibits *sinI* transcription, leaving SinR uninhibited and blocking matrix production in surfactin producing cells (figure 4).

This introduces a unidirectional path in biofilm development in *B. subtilis* creating a timing mechanism that plays a role in creating the various cell types of the bacterial culture.

Future research

Even though these publications show promising results for the clarification of the molecular mechanism behind surfactin immunity, it is important to note that no relation between the *srf* promoter, ComS and this gene expression was shown. Also, the expression of *eps* is blocked through the repression of *sinI* transcription, but it may be interesting to show that this indeed leaves the matrix producing genes inhibited.

In conclusion, the clarification of the mechanism of surfactin immunity reveals a step in bacterial development and gene regulation important for biofilm development in general, as well as timing in biofilm development.

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