On the function and interactions of bacterial flotillins

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Summary

Eukaryotic membrane compartments called lipid rafts are a well-understood structure, playing a role in a multitude of processes on the membrane surface. It was a fairly recent development that prokaryotes were found to have similar structures called functional membrane microdomains (FMMs). Functional in these FMMs are proteins called flotillins. These flotillins form homo-oligomers with high subunit turnover that are theorized to function as scaffolding proteins, specifically binding proteins which perform their function in FMMs. Flotillins also impact the fluidity of the cell membrane, although the exact mechanism with which this is done, is yet unknown. The two different flotillin proteins FloA and FloT in model organism *B. subtilis*, while both being flotillins, show vastly different characteristics. For one, the *floA gene* is constitutively expressed, while the *floT* gene is only expressed under starvation or end-of-growth conditions, FloA diffuses through the membrane around three times as fast as FloT, and FloA is markedly smaller than FloT. This essay compares the results of three different papers written about flotillins, concluding that what the literature described as potential binding partners either only interact for a very short time, or not at all.

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Introduction

The bacterial cell membrane is a vital component that separates the interior of the cell from the external environment. Composed of a phospholipid bilayer, the membrane accommodates integral and peripheral membrane proteins that regulate molecule transport and signaling. To facilitate the smooth operation of cellular processes and the organization of protein complexes on the outside of the plasma membrane, lateral diffusion of both lipid molecules and proteins within the plasma membrane is essential. This lateral diffusion relies for a large part on fluidity of the lipids in the membrane. This fluidity can be regulated by the cell through processes like selection of which lipids are incorporated into the membrane, or by incorporating certain membrane proteins into the plasma membrane.

A membrane protein, or more realistically, a multitude of membrane proteins, that affect membrane fluidity are flotillins. Flotillins are small, membrane-associated proteins that have been identified in both eukaryotic cells and bacteria. In eukaryotes, they exhibit a propensity to localize to cholesterol-enriched lipid microdomains known as lipid rafts (Simons et al. 1997), where they act as scaffolding proteins (Lopez & Koch 2017), recruiting other proteins to the lipid rafts. The eukaryotic flotillin proteins FLO-1 and FLO-2 bind to the membrane with their N-terminal domains (Neumann-Giesen et al. 2004) where they form heterooligomers with each other.

In bacteria, these flotillins are enriched in Functional Membrane Microdomains (FMMs), the bacterial homologs of lipid rafts (López et al. 2010), where they play an important role in membrane fluidity (Bach & Bramkamp 2013) and share with eukaryotic flotillins the function of acting as a scaffold for protein-protein interaction at the inner leaflet of the plasma membrane. An example of a protein-protein interaction that is facilitated by bacterial flotillins, is homodimerization of FMM-associated sensor kinases KinC and KinD, which are involved in biofilm formation in *B. subtilis* (Schneider et al. 2015). Another potential function of flotillins is prevention of non-specific protein-protein interactions.

Flotillins play diverse roles in cellular processes, including signal transduction, membrane trafficking, and cellular adhesion. In eukaryotes, they have been extensively studied, while in bacteria, their presence and functional significance are still being explored. Due to the comparative lack of study into bacterial flotillins, neither the precise pathways in which flotillins are involved, nor the exact mechanisms by which they accomplish these influences is precisely known. Research has been done, however, giving rise to speculative hypotheses, which will be outlined and compared in this paper.

Theorized functions and mechanics of flotillins

Flotillins functionally organize the bacterial membrane

A study done by Zielińska et al. (2020) aimed to determine what the effects of flotillins on plasma membranes are. They did this by first creating mutant strains of *B. subtilis* that had a deletion of the flotillin protein FloA, the flotillin protein FloT, or both of them alongside a deletion of a protein that is an essential component in a redundancy pathway for when the elongasome pathway no longer functions, pbp1. With these mutant strains, the effect of the absence of these proteins on cell morphology could be studied under a microscope. What this study found was that in the absence of both the two flotillins and pbp1, strong filamentation and delocalization of peptidoglycan synthesis was observed. This effect was also observed in the mutants that had only one flotillin deleted, albeit to a much lesser extent. Curious about this experiment was that when grown on growth media containing a limited supply of nutrients, none of the mutants showed a change in phenotype. Only when grown on rich medium did these changes manifest. This means that the phenotypic changes are either nutrient or growth rate-based.

When the changes in phenotype from the lack of flotillins were confirmed, the researchers shifted their focus to changes in lipid packing order in the membranes of these mutants with the use of Laurdan, a fluorescent dye used for staining the cell membrane to show the extent of lipid packing (Bach & Bramkamp, 2013). What they observed here was that, in line with the phenotypic changes outlined before, there were changes in generalized polarization, which points to an overall increase in ordered lipid packing in the membrane, suggesting that the presence of flotillins leads to an increase in ordered lipid packing. These changes manifested when the cells were grown on rich medium, but these changes disappeared entirely when grown on stringent medium. Of note here is that while there were clear changes observed in membrane fluidity, there were no changes found in overall fatty acid composition, meaning that the observed changes in phenotype were caused by a shift in fatty acid organization or a process akin to that. A bottleneck these researchers ran into was that with the resolution provided by Laurdan staining, local changes in lipid ordering could not be distinguished, only the overall lipid ordering was made visible.

With the knowledge that flotillin deletion leads to adverse effects in respect to peptidoglycan synthesis and other processes involved in cell division when grown on rich medium, alongside the discovery that an absence of flotillins leads to a less fluid membrane, the researchers looked into what the effects are of restoring membrane fluidity through the use of benzyl alcohol, a widely used membrane fluidizer. The results of this being that in the presence of benzyl alcohol, the membranes of the double flotillin mutant were restored in fluidity to levels similar to, or greater than, wildtype strains. With this restored membrane fluidity came normalization of cell length and peptidoglycan synthesis, suggesting that membrane fluidity, and with that the presence of flotillins, has an effect on these processes.

Protein complexes called MreB patches are involved in elongasome activity, moving around the membrane and delivering peptidoglycan synthesis proteins to the correct location. These patches function similarly in prokaryotes as actin does in eukaryotes, contributing to cell morphology and division Chastanet et al. (2012). Billaudeau et al. (2017) suggests that the speed of MreB patch movement is linked to the levels of nutrients available in the medium the bacteria (in the case of this study, *B. subtilis*) is grown on. What Zielińska et al. (2020) found, is that in the absence of flotillins, MreB patch mobility was greatly reduced in cells grown on rich medium, while in those grown on stringent medium, MreB patch mobility seemed unaffected by the lack of flotillins. When treated with benzyl alcohol, cells grown on rich medium had their MreB patch mobility restored to close to what it would be in the presence of flotillins. This suggests that MreB patch mobility is not only regulated by growth rate, but also by membrane fluidity, which results in a change in peptidoglycan synthesis in cells that grow fast and have a reduction in membrane fluidity. What this means in the context of flotillins, is that a lack of flotillins in fast-growing cells leads to a reduction in MreB movement, causing the previously observed changes in peptidoglycan synthesis, and with that, cell division.

Study into colocalization and protein-protein interactions of flotillins

A study done by Schneider, Klein et al. (2015) aimed to prove that the two different known flotillin proteins lead to the formation of two distinct types of FMMs. One of these families only harbors FloA and the proteins it acts as a scaffolding for, and the other type harbors both FloA and FloT with their associated proteins. The latter of these families only exists in the late stages in cell growth, having the function to adapt to the stationary phase where access to nutrients is limited.

To prove the existence of these two types, the researchers first had to prove that FloA and FloT are differentially regulated. They did this by creating a mutant *B. subtilis* strain that fused the promoters of the FloA and FloT proteins with a yellow fluorescent protein (YFP) reporter. They then grew these mutant strains on rich medium and on limiting medium. This resulted in the finding that *floA* expression was constant across the two media used, while the expression of *floT* was limited to the cells that were grown on limiting medium. This shows that the expression of *floT* only occurs when access to nutrients is limited. The researchers then went on to find out if this differential expression was a cell-regulated process. This was accomplished by systematically inactivating regulatory genes and measuring the effects on the expression of *floA* and *floT* when grown in limiting medium. This resulted in an expression of FloA that remained constant across all mutants, and two proteins that affected the expression of *floT*, being abrB, deletion of which leads to an increase in *floT* expression, and *spo0A*, deletion of which results in decreased *floT* expression. These proteins both belong to the same signaling pathway. *AbrB* is, among other functions, a repressor of biofilm formation and is negatively regulated by *spo0A*, which explains the results of deletion on the expression of *floT*.

The researchers then looked into how FloA and FloT interact with one another. This was done by performing a bacterial two-hybrid (BTH) assay, where a reporter gene gets activated upon

interaction of two tagged target proteins. When these two proteins interact, their tags combine into a transcription factor for a reporter gene, in the case of this assay this transcription factor modulates a *lacZ* gene. This assay was carried out multiple times, once tagging FloA with both parts, once tagging FloT with both parts, and once tagging FloA with one and floT with the other. This assay showed that in the case of only tagging FloA or only tagging FloT, a strong signal was observed, but when FloA and FloT were tagged with separate parts, a significantly weaker signal was observed. Another BTH assay was performed to determine the extent to which modifications in the glutamate alanine repeat (EA-repeat) regions in the C-terminus of the two flotillins (EA→GL), which was already found to be responsible for oligomerization of human flotillins, interfered with bacterial flotillin oligomerization. What these researchers found, was that with modifications in EA1, EA2, and EA4, homooligomerization was hindered significantly with FloA, with modifications in EA3 not seeming to have much of an effect, while with FloT, modifications in EA1 had a lesser effect than with FloA, while modifications in EA3 had more of an effect than with FloA.. The results of both BTH assays are shown in figure 1.

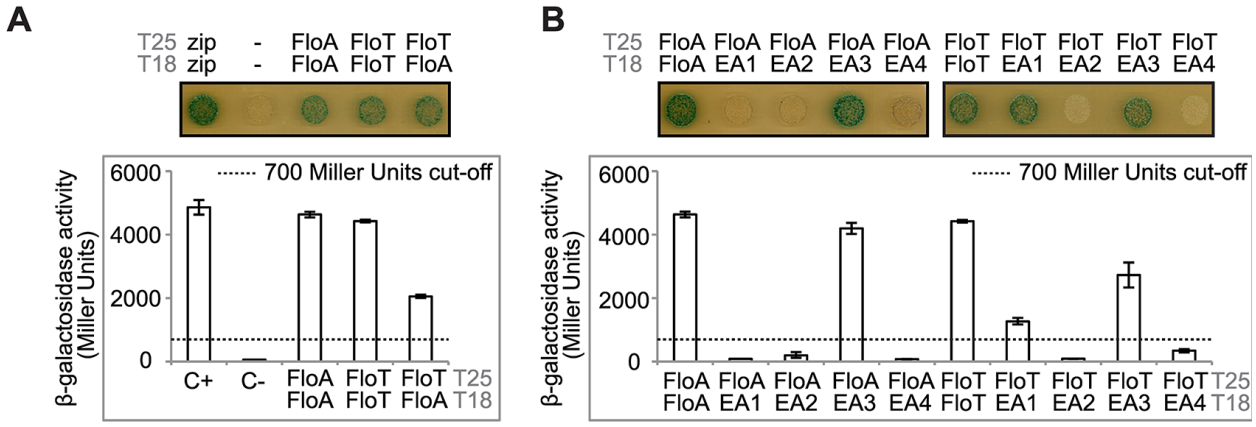

Figure 1: Results of the BTH assays performed by Schneider, Klein et al. (2015). Higher β-galactosidase activity points to a greater degree of interaction between the two tags. The dashed line represents the cutoff line for the Miller Units value from which the signal is significant. **A** In the case of FloA FloA and FloT FloT combinations, this signifies homodimerization, while the FloT FloA signifies heterodimerization. **B** Here, the EA-repeat regions in the C-terminus of FloA and FloT were individually modified.

The researchers then aimed to find out if the different spatio-temporal distribution patterns resulted from the difference in functionality between the two flotillin proteins, stemming from acting as a scaffold for the binding of proteins from distinct signal transduction pathways. To do this, the researchers expressed His^6-labeled FloA and FloT proteins in a strain of *B. subtilis*. The membrane fraction was then resolved using blue-native PAGE (BN-PAGE), which allows the proteins to remain in their non-denatured state during the PAGE. A Western blot was then performed on the gel resulting from this BN-PAGE using antibodies against His^6. Bands that showed up in the Western blot were then cut from the gel and analyzed with mass spectrometry (MS). MS analysis showed 9 membrane proteins that the researchers concluded to be exclusively associated with FloA, including cytoskeletal-associated proteins MreC and PBP1A/1B, and proteins related to cell wall remodeling like TagU and PhoR. The binding to MreC lines up with the influence of flotillins on MreB movement discovered by Zielińska et al. (2020). The researchers decided to look further into the interaction between FloA and PhoR, as

PhoR is a component in the signaling cascade that leads to cell wall organization. A BTH assay showed an interaction between PhoR and FloA that was not observed between PhoR and FloT. MS-analysis also showed a multitude of potential interacting partners to FloT, including some that were theorized to be potential interacting partners in previous studies, like Bach & Bramkamp (2013). A notable protein here is the membrane-bound sensor kinase ResE. A BTH assay showed an interaction between ResE and FloT that was not observed between ResE and FloA.

The interactions between FloA and PhoR and between FloT and ResE were studied further by colocalization experiments using FloA-mCherry, PhoR-GFP and FloT-mCherry, ResE-GFP double-labeled strains. Colocalization was then measured using fluorescence microscopy, showing a Pearson's correlation coefficient of $R^2 = 0.82$ for FloA-PhoR and $R^2 = 0.85$ for FloT-ResE. From this, it can be concluded that PhoR and ResE are spatially correlated with their respective flotillins. This leads to the generation of a model that suggests that FloA and FloT have distinct interaction characteristics and the tethering of different signal transduction pathways leads to the control of different cellular processes.

FloA and FloT form dynamic structures and move through the membrane at different speeds

A study done by Dempwolff et al. (2016) aimed to gain more clarity on how flotillin proteins move through, and interact in, the membrane. These researchers first performed microscopy using Stimulated Emission Depletion (STED) superresolution microscopy. They labeled FloA and FloT with YFP in *B. subtilis*. From this microscopy followed that the foci of the FloT assemblies had an average size of 85.3 nm ± 12.5 nm with a maximum of 97 nm, while the FloA assemblies had an average size of 80.4 nm ± 10.9 nm. What the researchers concluded from this is that flotillin assemblies have a preferred size that differs between the two flotillin proteins.

The researchers then turned their attention to the oligomerization of the flotillin proteins. They purified the soluble part of FloA through the use of a Ni-NTA column using His^6 as an affinity tag. They followed this up with analytical gel filtration (GF) and sucrose gradient centrifugation. These separation methods resulted in a high molecular weight (HMW) peak of 670 kDa, and another lower molecular weight (LMW) fraction with a peak around 60 kDa, which corresponds to the weight of a FloA dimer. This suggests a tendency of FloA to form homo-oligomers with itself. To ascertain that the His^6 affinity tag was not influencing the formation of oligomers, another purification was performed using a strep-tag. This resulted in similar results with the only difference being that the low molecular weight fraction was smaller, and had a peak at 120 kDa, corresponding to a FloA tetramer as its smallest assembly. GF analysis on 3- and 5-fold dilutions of the HMW fraction resulted in the observation that in the 3-fold dilution, instead of nearly everything being in the HMW fraction, only approximately 90% was in the HMW fraction, with the rest being in the LMW fraction, while in the 5-fold dilution, the numbers were 82% HMW and the rest LMW. A 2-fold concentration was also analyzed with GF, which resulted in an increased formation of multimers up to 670 kDa. These results combined suggest that there is an equilibrium between the smallest unit and multimers, with the equilibrium lying towards the

multimers, which in turn suggests that these multimers are fairly stable structures. This also means that flotillin structures are modulated through flotillin self-interaction.

To find out if these flotillin multimers are static in composition, or if the monomers they consist of are constantly exchanging, the researchers bleached fluorescent foci of FloT-YFP gradually until a single fluorescent spot is left, revealing the intensity of a single chromophore. From this, the number of single subunits can be approximated. After the number of subunits was approximated, the foci were left to regain fluorescent capabilities. This occurred over 100-150 frames, corresponding to around 2-3 seconds. This suggests that bleached fluorophores are replaced by non-bleached ones in the span of a few seconds, indicating high turnover.

An experiment into the dynamics of the movement of flotillin proteins through the membrane using a timelapse with 1 second intervals resulted in clearly distinguishable dynamics of FloA and FloT, having FloT move markedly slower than FloA, with FloA having a diffusion rate of 0.0056 $\mu m^2/s$ and FloT having a diffusion rate of 0.0018 $\mu m^2/s$, making FloA approximately 3 times as fast as FloT. From this difference in diffusion speed along the membrane, it can be concluded that FloA and FloT do not form a common structure, as the different speeds do not allow more than a few milliseconds for the different flotillins to interact with each other. The same was found for proteins that other literature like Schneider, Klein et al. (2015) found to potentially interact with flotillins, like KinC, FtsH, SecA, ResE, PhoR and OppA. These proteins also move through the membrane with such differing velocities that interaction for more than a few milliseconds is unlikely.

Conclusion

This paper has outlined the results of three papers about the properties and functions of flotillins. The first of these papers went about probing the functions of flotillins by growing *B. subtilis* that lack flotillins and observing changes in phenotype under the absence of flotillins. They found that the changes in phenotype were reverted when the cells were treated with the membrane fluidizer benzyl alcohol, suggesting that flotillins are in some way involved in membrane fluidity.

The second of these papers first used fluorescence microscopy to show that FloA expression happened when nutrients were abundant, as well as when nutrients are scarce, while FloT expression only happened when nutrients were scarce. When looking deeper into this differential expression by systematically inactivating regulatory genes, the observation was made that the regulatory genes abrB and spo0A deletions affected FloT expression. They also performed a set of BTH assays that showed that the flotillin proteins interact strongly with themselves, but more weakly with each other. These assays also showed that changes in some of the EA repeat regions of the C-terminus of flotillins lead to significantly reduced self-interaction. Lastly, they performed a BN-PAGE, followed by a Western blot with antibodies against a His^6-tag that was attached to the flotillins, from which the bands that appeared were extracted and analyzed with MS. This resulted in a set of membrane proteins that was deemed

to exclusively interact with FloA and another set that was deemed to exclusively interact with FloT.

The last of the papers discussed looked mostly into the movement and interaction of the two flotillins within the membrane. The researchers first performed STED superresolution microscopy, with which they measured the size of foci from YFP-labeled FloA and FloT, from this, it was observed that FloA and FloT have a different, but constant, preferred size to their assemblies. The researchers then purified His^6-labeled FloA with a Ni-NTA column and weight-separated this purified product. This led to the observation that most of this product had a molecular weight of 670 kDa, while a small fraction had a molecular weight of 60 kDa. The 60 kDa fraction corresponded to a FloA dimer, and the 670 kDa fraction to a multimer. The researchers aimed to find out if this 670 kDa structure is static or dynamic in its constituents, so they photobleached some of these foci and observed how long, if at all, it would take for the foci to regain some fluorescence. The result of this was that it takes around 2-3 seconds for the foci to regain fluorescence, suggesting that the multimers have a high turnover. Lastly, they looked at how fast the proteins moved along the membrane, and found that FloA moves around 3 times as fast through the membrane as FloT, suggesting that FloA-FloT interaction can only be a few milliseconds.

Something else of note in these papers is the result generated by the BN-PAGE experiment from the second paper. This experiment did generate bands on the PAGE gel that did actually contain labeled proteins for the Western blot. But what these bands likely also contained, is other, unrelated proteins or protein complexes that share the molecular weight with the protein complexes containing the labeled proteins. The results from this experiment therefore had insufficient controls in place to generate scientifically significant results and any conclusions derived from it should be discarded.

Most of the conclusions from these three papers can coexist without contradictions. The exception to this are the conclusion of the BTH assay from the second paper, which suggests that FloA and FloT do form hetero-oligomers, albeit less strongly than homo-oligomers, and the last conclusion of the third paper, which suggests that due to their vastly differing diffusion speeds, cannot interact for more than a few milliseconds. This contradiction means that more study into the matter with more diverse methods is required to draw any kind of robust conclusions.

What can be derived from these papers, is that bacterial flotillins are membrane proteins that form homo-oligomers, as well as to a lesser degree hetero-oligomers, which both have high subunit turnover. These proteins seem to move laterally through the membrane bound to MreB patches, where they influence elongasome activity. They also have an impact on membrane fluidity, but the exact mechanism how they accomplish this is yet unknown. And lastly, the exact set of proteins the flotillins interact with still lack scientific consensus. For this reason, further study into these unknowns is a good next step.

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