Overproduction of Flotillin Influences Cell Differentiation and Shape in *Bacillus subtilis*

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**ABSTRACT** Bacteria organize many membrane-related signaling processes in functional microdomains that are structurally and functionally similar to the lipid rafts of eukaryotic cells. An important structural component of these microdomains is the protein flotillin, which seems to act as a chaperone in recruiting other proteins to lipid rafts to facilitate their interaction. In eukaryotic cells, the occurrence of severe diseases is often observed in combination with an overproduction of flotillin, but a functional link between these two phenomena is yet to be demonstrated. In this work, we used the bacterial model *Bacillus subtilis* as a tractable system to study the physiological alterations that occur in cells that overproduce flotillin. We discovered that an excess of flotillin altered specific signal transduction pathways that are associated with the membrane microdomains of bacteria. As a consequence of this, we detected significant defects in cell division and cell differentiation. These physiological alterations were in part caused by an unusual stabilization of the raft-associated protease FtsH. This report opens the possibility of using bacteria as a working model to better understand fundamental questions related to the functionality of lipid rafts.

**IMPORTANCE** The identification of signaling platforms in the membrane of bacteria that are functionally and structurally equivalent to eukaryotic lipid rafts reveals a level of sophistication in signal transduction and membrane organization unexpected in bacteria. It opens new and promising venues to address intricate questions related to the functionality of lipid rafts by using bacteria as a more tractable system. This is the first report that uses bacteria as a working model to investigate a fundamental question that was previously raised while studying the role of eukaryotic lipid rafts. It also provides evidence of the critical role of these signaling platforms in orchestrating diverse physiological processes in prokaryotic cells.

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Bacterial membranes are composed of different types of lipids, which tend to aggregate according to their physicochemical properties and accumulate into lipid domains that are immiscible with the surrounding lipids (1). The heterogeneous organization of membrane lipids leads to a lateral segregation of the embedded membrane proteins, which seems important for their functionality (2). One of the most interesting examples of the heterogeneous segregation of lipids and proteins is the formation of functional microdomains in the membrane of bacteria that are structurally and functionally equivalent to the lipid rafts of eukaryotic cells (3–5). Bacterial microdomains are membrane platforms that organize a group of proteins related to signal transduction and protein secretion (6). The integrity of these signaling platforms is essential for the correct functionality of the harbored proteins. Consequently, any alteration in their architecture severely inhibits the physiological processes related to the harbored proteins, such as biofilm formation, motility, competence, or protease secretion (6, 7).

The integrity of bacterial lipid rafts relies on the biosynthesis and aggregation of polyisoprenoid lipids and the presence of flotillin proteins (6, 8). Flotillins are membrane-bound proteins that localize exclusively in the lipid rafts and are usually considered a bona fide marker for the localization of lipid rafts. The function of flotillins in lipid rafts is not entirely understood, yet it is believed that they may act as chaperone proteins to recruit protein cargo to lipid rafts and facilitate interactions and oligomerization (9–12). Hence, the presence of flotillin in lipid rafts is necessary for the correct functionality of the associated signaling processes. In eukaryotic cells, alterations in the functionality of flotillins often occur in association with severe physiological dysfunctions in cells (13). For instance, the development of Alzheimer’s disease or Parkinson’s disease is usually observed in cells that concomitantly overproduce flotillin proteins (14, 15), as well as in neuronal cells with severe lesions (16, 17). Despite this interesting correlation, it is still unclear whether the overproduction of flotillin contributes to the physiological alterations or is actually a consequence of the disease. The number of technical challenges associated with the manipulation of eukaryotic cells has complicated the study of the role of flotillins (18). This motivated us to use the bacterium *Bacillus subtilis* as a working model to evaluate whether the overproduction of flotillins causes any alteration in the cellular physiology of the bacterium and whether this effect could possibly result in cellular dysfunction.

The functional membrane microdomains of the bacterial model organism *Bacillus subtilis* contain two structurally similar flotillin-like proteins, which are referred to as FloA (formerly...
YqfA (6, 19) and FloT (formerly YuaG) (6, 8). Cells lacking FloA and FloT are defective in a number of signal transduction pathways that are associated with the protein cargo of the functional membrane microdomains (e.g., biofilm formation, sporulation, motility, or competence) (6, 8, 20, 21). Thus, it is believed that FloA and FloT facilitate the interaction and oligomerization of the protein cargo in the functional microdomains of \( B. \ subtilis \) in a fashion similar to that described for eukaryotic lipid rafts. Supporting this hypothesis, a direct interaction of FloA and FloT with the protein cargo protease FtsH (22) has been reported that is important for the protease activity of FtsH. Furthermore, an additional number of FloT-interacting proteins have been identified recently, including a number of proteins related to signal transduction and protein secretion (20). Accordingly, protein secretion was reduced in cells lacking flotillin, which suggests that the associated protein secretion machinery loses its functionality in the absence of flotillin (20).

The first signal transduction pathway described in association with the flotillins of \( B. \ subtilis \) leads cells to specialize in the production of extracellular matrix to ultimately form biofilms (6). The induction of this signaling pathway is driven by the activation of the master regulator Spo0A via phosphorylation (\( \text{Spo0A}^\text{P} \)) (23, 24). The membrane-bound sensor kinase KinC, responsible for phosphorylating \( \text{Spo0A}^\text{P} \), is part of the protein cargo, and its activity depends on the functionality of FloA and FloT (6) in a similar manner to the protease FtsH (22). FtsH is responsible for degrading the phosphatase enzymes that ultimately inactivate \( \text{Spo0A}^\text{P} \) by dephosphorylation (25) and actively contributes to the differentiation of matrix-producing cells (22). Therefore, published data suggest that the signal transduction pathway that is involved in biofilm formation is controlled by regulatory proteins, which localize in the functional membrane microdomains.

Further evidence was presented that exponentially growing \( B. \ subtilis \) cells accumulate FloA and FloT in the septum of dividing cells (22), suggesting that flotillins interact with septum-localized proteins, with FtsH as one example of this kind. This suggests a possible involvement of flotillins in processes related to cell division or cell shape. Related to this observation, other laboratories have determined that \( B. \ subtilis \) cells lacking flotillins underwent an aberrant cell division process (7). Thus, these two physiological features involving cell division and biofilm formation seemed affected by FloA and FloT in \( B. \ subtilis \) cells and could be studied to monitor the functionality of flotillins in \( B. \ subtilis \) cells.

In this report, we show that a 5-fold induction in the production of FloA and FloT significantly increased the amount of flotillin harbored in the microdomains, and this severely affected biofilm formation and cell division in \( B. \ subtilis \). The subpopulation of cells specialized to produce extracellular matrix increased due to an implementation of FtsH activity. Cells overproducing FloA and FloT showed a more efficient septation process, which resulted in shortened cells, minicells, and cells with aberrant septa. As a consequence of the FloA and FloT overproduction, an implementation of the FtsH protease activity occurs, which negatively affects the stability of the protein EzrA (Extra Z-rings assembly), an inhibitor of septum formation (26–28). Altogether, overproduction of flotillins severely affected important cellular processes that directly impacted the physiology of the cells and could potentially contribute to the development of severe diseases in other living organisms.

**RESULTS AND DISCUSSION**

The overproduction of FloT and FloA reached saturation in \( B. \ subtilis \). The bacterial model \( B. \ subtilis \) was used to overexpress the floA and floT genes under the control of an isopropyl-\( \beta \)-d-thiogalactopyranoside (IPTG)-inducible promoter, \( p_{\text{hp}} \) (the Hyperspank promoter). To first compare the overexpression levels to the natural expression levels, we generated translational fusions of floA and floT to a green fluorescent protein (GFP) gene (\( \text{gfp} \)) anchored to the cytosolic C-terminal part of the protein. PY79 \( p_{\text{floA-GFP}}, p_{\text{floT-GFP}}, p_{\text{floA-GFP}}, \) and \( p_{\text{floT-GFP}} \)-labeled strains were grown in liquid cultures of the chemically defined medium M5gg at 30°C until they reached the stationary phase (29). Cells were harvested, fixed with paraformaldehyde, and examined with a fluorescence microscope. Expression from the native promoter showed that the fluorescence signal attributable to FloA and FloT was organized as discrete foci across the cellular membrane and was occasionally positioned in the septum of dividing cells, as previously reported (Fig. 1A and B) (22). The strains expressing \( p_{\text{floA-GFP}} \) and \( p_{\text{floT-GFP}} \) constructs showed expression levels below the natural level of expression in the absence of IPTG (see Fig. S1 in the supplemental material).

Indeed, the addition of IPTG to the cultures induced the expression of FloA and FloT. Fluorescence microscopy analysis showed that 0.1 mM IPTG resulted in expression levels comparable to those of the native promoters (see Fig. S1). Saturation of flotillin levels was achieved by adding 15 mM IPTG to the cell cultures. No significant changes in the expression of FloA or FloT were detected in cells that grew with IPTG concentrations above 15 mM. The presence of IPTG did not cause any significant growth alteration in cultures of the PY79 strain (see Fig. S2 in the supplemental material).

Next, we compared the increment in the relative fluorescence signal between native expression and IPTG-inducible full expression. On average, our results showed that full induction by IPTG caused an approximately 5-fold increase in the production of FloA and FloT fluorescence signals in comparison to the native expression level. Further examination at the single-cell level showed that the induction of FloA and FloT expression resulted in an increase of the fluorescence intensity of the membrane foci and, to a lesser extent, in the number of foci. Figure 1C and D show the analysis of the subcellular organization of the fluorescence signal in representative single cells expressing FloA-GFP and FloT-GFP at native and full induction levels, respectively.

**Saturation of FloT and FloA affects biofilm formation and cell differentiation.** Biofilm formation in \( B. \ subtilis \) requires the differentiation of numerous cell types. Among those, the matrix-producing cell type is responsible for the production and secretion of the extracellular matrix of the biofilm (24, 30, 31). The subpopulation of matrix producers simultaneously expresses the \( \text{epsA-O} \) operon (henceforth called the \( \text{eps} \) operon) and the \( \text{tapa}-\text{sipW-tasA} \) operon (henceforth called the \( \text{tasA} \) operon). The \( \text{eps} \) operon encodes the enzymes responsible for the production of the extracellular exopolysaccharide (32–34). The \( \text{tasA} \) operon is required for the production of extracellular amyloid fibers that structurally give consistency to the biofilm (35–39).

To investigate whether the overexpression of FloA and FloT affects biofilm formation, strains overproducing FloA and FloT were constructed and used to assay biofilm formation. The morphology of \( B. \ subtilis \) colonies grown on solid biofilm-inducing
MSgg agar medium was examined. To generate biofilms on agar, 3 µl of an LB preculture was spotted on MSgg medium supplemented with 15 mM IPTG and allowed to grow at 30°C for 72 h. After incubation, the biofilms develop into integrated microbial communities with great complexity, manifested by the number of wrinkles present on the surface of the biofilm, which is representative of the robustness and the consistency of the extracellular matrix of the biofilm. Two different genetic backgrounds were used to perform this assay—the PY79 and NCIB3610 strains. Strain PY79 is a laboratory strain that is partially deficient in biofilm formation due to the acquisition of a point mutation in the eps operon (Fig. 2A) (40). While this strain still possesses an intact tasA operon, the lack of extracellular polysaccharide makes the PY79 microbial communities flat, and they lack any distinctive complex architecture (40). A deletion of the transcriptional repressor SinR, which uncouples the regulation of the biofilm (33, 41), resulted in a slightly wrinkled colony even in the absence of a functional eps operon, suggesting that overexpression of the TasA strain partially restored biofilm formation when overproduced in the PY79 strain. Accordingly, the double mutant ΔsinR ΔtasA strain showed a biofilm-null phenotype, suggesting that biofilms generated by PY79 are mainly caused by expression of TasA or the eps operons significantly reduce the ability of this strain to form biofilm, and a depletion of SinR results in a hyperwrinkled colony that overproduces extracellular matrix (see Fig. S3 in the supplemental material).
Overexpression of the flotillin-like proteins FloA and FloT promotes biofilm formation via FtsH. (A) Schematic representation of how FloA and FloT influence the signaling pathway leading to matrix production. Spo0A is activated by phosphorylation (Spo0A~P) and indirectly derepresses the expression of the eps and tapA operons. Phosphorylation of Spo0A~P is driven by the action of the membrane-bound kinase KinC, which is stabilized by FloA and FloT. Spo0A~P is subjected to dephosphorylation by a pool of phosphates that are degraded by the protease FtsH. The activity of FtsH is dependent on FloA and FloT. Solid arrows denote direct regulation, and dashed arrows denote indirect regulation. The asterisk denotes mutations in PY79. (B) Colonies of diverse mutants in the PY79 strain grown in MSgg agar medium for 72 h at 30°C. (C) Effects of flotillin overexpression in colony morphology of PY79 grown on solid MSgg agar medium for 72 h at 30°C with 15 mM IPTG. The scale bar represents 0.5 cm. (D) Flow cytometry analysis to monitor the subpopulation of matrix-producing cells in different genetic backgrounds using the P_{tapA-yfp} reporter system. Cells were grown for 72 h on solid MSgg medium in the presence of 15 mM IPTG. Approximately 50,000 ungated cells were analyzed. Fluorescence is represented in arbitrary units (AU). ctrl, control; wt, wild type. (E) Diverse mutants in the PY79 strain, we used this genetic background to further explore the molecular effects in cell differentiation associated with the overproduction of FloA and FloT in *B. subtilis.*

Strains expressing P_{tapA}FloA, P_{tapA}FloT, and P_{tapA}FloA P_{tapA}FloT were constructed in different genetic backgrounds. The resultant strains were assayed for variations in their ability to form biofilm by allowing them to grow in agar MSgg medium supplemented with 15 mM IPTG at 30°C for 72 h. After incubation, the biofilms of the strains overexpressing FloA or FloT were morphologically indistinguishable from the wild-type strain, indicating that the overexpression of a single flotillin protein did not influence biofilm formation (Fig. 2C). However, the P_{tapA}FloA P_{tapA}FloT strain that simultaneously overexpressed FloA and FloT resulted in a more robust, biofilm-like colony morphology that was especially evident in the experiments that used the PY79 strain (Fig. 2C; see Fig. 5 in the supplemental material to compare it to other genetic backgrounds). Because PY79 harbors a non-functional eps operon, we reasoned that the biofilm formation phenotype observed in the PY79 P_{tapA}FloA P_{tapA}FloT strain was attributable to the overexpression of TasA protein and the high production of amyloid fibers. A similar effect was observed in the experiments that used the 3610 strain, although this genetic background showed milder effects for reasons that are unknown to us. Hence, based on the robustness and the consistency of the biofilm formation phenotype observed in the PY79 strain, we used this genetic background to further explore the molecular effects in cell differentiation associated with the overproduction of FloA and FloT.
TasA, which ultimately induced biofilm formation in the PY79 strain.

**Overproduction of flotillin stimulates FtsH activity.** The genetic cascade responsible for the differentiation of matrix producers is triggered by phosphorylation of Spo0A–P. The membrane kinase KinC induces Spo0A–P phosphorylation in response to the secretion of the signal surfactin (Fig. 2A) (42). Moreover, the activity of the membrane-bound protease FtsH is equally important for matrix production, because FtsH degrades the phosphatases that are responsible for the deactivation of Spo0A–P by dephosphorylation (25). Importantly, both KinC and FtsH proteins localize to the functional membrane microdomains in *B. subtilis*, and their functionality is dependent on the activity of FloA and FloT (6, 22). Figure 2A shows an overview of the regulatory cascade leading to biofilm formation.

We hypothesized that the molecular mechanism underlying the evident increase in the subpopulation of matrix producers could be related to the positive effects of FloA and FloT on the activity of KinC or FtsH. Importantly, the PY79 strain is not able to produce surfactin due to the acquisition of a point mutation in the *sfp* gene during laboratory domestication (Fig. 2A) (40). The Sfp protein is a phosphopantetheinyl transferase that posttranslationally modifies the surfactin biosynthesis machinery. This is an essential process for the correct functionality of the surfactin biosynthesis machinery (43, 44). Thus, the activation of KinC via surfactin is not possible in the PY79 strain. This fact led us to focus on the activity of the membrane-bound protease FtsH.

FtsH indirectly affects the levels of Spo0A–P by degrading four regulatory phosphatase proteins, RapA, RapB, RapE, and Spo0E, which feed into the Spo0A phosphorelay to ultimately decrease the levels of Spo0A–P. The absence of FloA and FloT negatively affects the FtsH protein (22), which prevents the degradation of the RapA, RapB, RapE, and Spo0E phosphatases (25). To explore whether the overproduction of FloA and FloT decreased the levels of Spo0A–P via FtsH, we deleted the *ftsH* gene in the wild-type and *P*<sub>hp</sub>*FloA P*<sub>hp</sub>*FloT strains, and we monitored biofilm formation. The ∆*ftsH* and ∆*ftsH P*<sub>hp</sub>*FloA P*<sub>hp</sub>*FloT strains were grown in MSgg agar medium supplemented with 15 mM IPTG and were incubated at 30°C for 72 h. After incubation, the microbial communities of the ∆*ftsH* P<sub>hp</sub>*FloA P<sub>hp</sub>*FloT strain showed no particular biofilm architecture but a flat morphology comparable to that of the wild-type and ∆*ftsH* strains (Fig. 2E). Next, these strains were labeled with the *p*<sub>mpA</sub>–*yfp* transcriptional fusion to monitor possible variations in the subpopulation of matrix producers by using flow cytometry. Flow cytometry analysis showed that the overproduction of FloA and FloT did not differentiate the subpopulation of matrix producers in cells lacking FtsH (Fig. 2F). This suggests that FtsH mediated the differentiation of matrix producers when FloA and FloT were artificially overproduced.

These results are in agreement with published literature showing that the oligomerization of FtsH in *E. coli* requires the chaperone activity of HIC and Hfk, two proteins that are structurally similar to FloA and FloT (45–48). *B. subtilis* lacks the HIC and Hfk proteins, and thus, it is possible that FloA and FloT might play the role of HIC and Hfk in stabilizing FtsH in *B. subtilis*. To test this hypothesis, whole-cell extracts of the *P*<sub>mp</sub>*FloA P*<sub>mp</sub>*FloT strain were used to semiquantitatively detect FtsH by immunoblot analysis using polyclonal antibodies against FtsH. An increase in FtsH protein was detected in normalized cell extracts from the *P*<sub>hp</sub>*FloA P*<sub>hp</sub>*FloT strain compared to the wild-type strain (Fig. 3A). Next, we observed that these higher levels of FtsH coincided with higher levels of TasA. We performed an immunoblot analysis using polyclonal antibodies against TasA. The extracts of cells that overproduced FloA and FloT, which showed higher levels of FtsH, also showed higher levels of TasA. Importantly, when the *ftsH* gene was deleted, the detection of TasA was not possible in the strain that overproduced FloA and FloT (Fig. 3B).

Altogether, the data are consistent with the hypothesis that the functional link between FloA/FloT and FtsH mediated the increase of the subpopulation of matrix-producing cells in the PY79 strain that overproduced FloA and FloT. This, in turn, caused an increase in the production of TasA, which resulted in an overproduction of biofilm formation. In those lines, the interaction of FtsH-like proteins with flotillin-like proteins has been described in many systems and organelles (e.g., mitochondria, yeast, or plants), and it has been shown that the stability of FtsH-like proteins depends on the presence of flotillin-like proteins (49, 50). It is hypothesized that the chaperone activity of the flotillin-like proteins acts as a regulator to fine-tune the proteolytic activity of FtsH (49, 50). It also is suggested that flottillins serve as scaffolding proteins to limit the mobility of the FtsH protease across the membrane (49). Based on these current hypotheses, it is probably not surprising that the overproduction of FloA and FloT affected the activity of FtsH of *B. subtilis*.

**Flotillin overexpression results in decreased cell length.** FtsH principally localizes to the septum of dividing cells (51), where the interaction with FloA and FloT presumably occurs (22). Possibly, flottillins provide stability to protein septum-associated proteins. Accordingly, there is evidence of a significant number of septum-localized proteins among the interactome of FloT (20, 22). Furthermore, the absence of flottillins in *B. subtilis* has been associated with pleiotropic effects on cell shape (7), which led us to reason that septum-localized membrane microdomains could influence septum-associated processes, like septum formation or shape determination.

Septum formation and cell shape determination were analyzed at the single-cell level in cells that overproduced flottillins. To do this, the *P*<sub>hp</sub>*FloA, *P*<sub>hp</sub>*FloT, and *P*<sub>hp</sub>*FloA *P*<sub>hp</sub>*FloT strains were grown in liquid MSgg medium plus IPTG (15 mM) at 37°C with vigorous agitation until the late exponential growth phase (optical density at 600 nm [OD<sub>600</sub>] of 0.8 to 1.0). After incubation, cells were stained with the membrane dye FM4-64 before examination under the fluorescence microscope. We observed that under these growth conditions, the simultaneous overexpression of FloA and FloT resulted in a dramatic reduction of cell length (Fig. 4A, column 4; see Fig. S4 in the supplemental material). We randomly selected 500 cells from each microscopic field and measured the cell length (Fig. 4B). On average, wild-type cells showed a cell length of 2.41 ± 0.52 μm. This result was comparable to the length of cells expressing FloA (2.47 ± 0.60 μm) or FloT (2.34 ± 0.65 μm). However, overproduction of FloA and FloT resulted in a cell length of 1.11 ± 0.52 μm. Probably as a consequence of the reduction of cell length, *P*<sub>hp</sub>*FloA P<sub>hp</sub>*FloT cells also showed a partial loss of the typical *B. subtilis* rod shape, and a subtraction of cells (approximately 19% of the total) became spherical. Among those, we detected a significant number of small, circular, anucleate minicells (approximately 28% of the total) (52–54), as well as small, spherical cells that contained DNA (72% of the total).

The effect on cell length associated by the overexpression of FloA and FloT pointed to an influence of flottillins on the efficiency
of septum formation. To investigate the influence of FloA and FloT in the assembly of proteins with a relevant role in septum formation, we chose the FtsZ protein as a septum-related protein to monitor septum formation in *B. subtilis* cells. FtsZ forms a ring structure (Z-ring) to ultimately drive cell septation (55, 56), and mutants showing an increase in FtsZ assembly also showed additional division events per cell cycle, with the formation of extra septa that led to the occurrence of a minicell-like phenotype (55, 57). Thus, the phenotypic similarities between cells overexpressing FloA and FloT and cells with increases in FtsZ assembly led us to hypothesize a functional connection between these two genotypes. We first tested whether the overproduction of FloA and FloT affects the septation efficiency. To do this, cultures of the P<sub>xyl</sub>FloA P<sub>xyl</sub>FloT mutant were grown in liquid MSgg medium resistant membrane [DRM] fraction). It is known that the DRM fraction is enriched in proteins associated with lipid rafts (4, 59, 60) and is the fraction that contains the proteins of the functional membrane microdomains when assayed with *B. subtilis* membranes. We detected the protein EzrA in the DRM fraction of *B. subtilis*, with identification coverage of 53% (see Fig. S5 in the supplemental material) (22). EzrA is a negative regulator of the assembly of the Z-ring that localizes in the midcell in an FtsZ-dependent manner. Cells depleted of EzrA (Extra Z-rings assembly) show multiple Z-rings located at the polar and medial sites (27, 61). We hypothesized that overproduction of FloA and FloT could negatively affect the activity of EzrA to promote an accelerated assembly of FtsZ.

To address this question, we generated P<sub>xyl</sub>EzrA-GFP and P<sub>xyl</sub>FloA P<sub>xyl</sub>FloT P<sub>xyl</sub>EzrA-GFP strains to compare the levels of expression of EzrA in cells overexpressing FloA and FloT. Examination of cells under the fluorescence microscope did not render conclusive results. Instead, we performed a semiquantitative im-

![Image 1](https://example.com/image1)

**FIG 3** Overproduction of FloA and FloT increase the levels of FtsH and TasA proteins. (A) Immunoblot analysis to detect FtsH in different mutants using polyclonal antibodies against FtsH. The wild-type strain is used as a positive control, while the ΔftsH mutant is used as a negative control. The immunoblot signal is presented in the upper panel, and the respective SDS-PAGE result is presented in the lower panel as a loading control. (B) Detection of TasA production in different mutants by immunoblot analysis using polyclonal antibodies against TasA. The wild-type strain (PY79) is used as a positive control, while the ΔtasA mutant is used as a negative control. The immunoblot signal is presented in the upper panel, and the respective SDS-PAGE result is presented in the lower panel. Samples were obtained from the extracellular protein fraction of pellicles that were grown in liquid cultures for 24 h at 30°C. Protein levels were normalized relative to cell number.
munoblot assay using polyclonal antibodies against GFP. Using this assay, we observed a significant reduction of EzrA levels in the extracts of the PhpFloA PhpFloT PxylEzrA-GFP strains in comparison to the P xylEzrA-GFP strain (Fig. 6A). Although this result explained the higher efficiency to form Z-rings and septates in cells overproducing FloA and FloT, it was somewhat unexpected, as one might anticipate that an enhanced chaperone activity of flotillins should always affect the stability of the associated proteins in a positive fashion. One plausible hypothesis that could explain this result is that additional proteins that are stabilized by FloA and FloT negatively influence EzrA. Supporting this hypothesis, we found evidence in the literature that EzrA of *B. subtilis* is degraded by an ATP-dependent protease that is structurally similar to FtsH (62), suggesting that FtsH could target EzrA in the strain that overexpressed FloA and FloT. This is consistent with the filamentous growth that is described in the mutant lacking FtsH, which is also observed in strains with defective cell septation (63). Consequently, we tested whether FtsH influenced the reduced levels of EzrA observed in cells overexpressing FloA and FloT. To do this, the levels of EzrA-GFP were tested in the presence or absence of FtsH by semiquantitative immunoblot assays, using whole-cell extracts of the P _hp FloA P _hp FloT P _sy EzrA-GFP and ΔftsH P _hp FloA P _hp FloT P _sy EzrA-GFP strains. Using this approach, we observed that cells lacking FtsH showed increased lev-
els of EzrA (Fig. 6B). Moreover, we generated the PhpFtsH PxylEzrA-GFP strain, which overexpressed PhpFtsH, and whole-cell extracts of this strain were used to compare the abundance of EzrA before and after FtsH overproduction. Figure 6B shows that the overproduction of FtsH was associated with reduced EzrA levels. We detected an intriguing protein band in this strain that could be attributed to an alternative processed form of EzrA protein. More experiments should be performed in this direction to fully address whether a direct interaction exists between FtsH and EzrA. Experiments presented in this report are consistent with the idea that the overexpression of flotillins causes severe physiological changes in bacterial cells.

Overall, our study showed that overexpression of FloA and FloT in the functional membrane microdomains of B. subtilis resulted in severe defects in cell differentiation and cell shape, as illustrated in Fig. 7. It is probably not surprising that this important regulatory node localizes in the functional membrane microdomains, under the direct control of the two flotillin-like proteins FloA and FloT, in a manner similar to that found in other biological systems (49, 50). Yet, when focusing on B. subtilis, it is still unknown whether both FloA and FloT play redundant functions in the functional microdomains. Further experiments are necessary to clarify why the overexpression of two structurally different flotillin proteins is required to achieve the described effects. We tend to think that FloA and FloT are partially redundant and some physiological processes are functionally linked to both FloA and FloT, while other physiological processes are specifically linked to FloA or FloT.

MATERIALS AND METHODS

Strains, media, and culture conditions. For general purposes, the B. subtilis strains PY79 and NCIB3610 were used in this study. Escherichia coli DH5α was used for cloning purposes. A detailed list of the genetically
modified strains is shown in Table S1 in the supplemental material. For routine growth, cells were propagated on LB medium. Selective media were prepared in LB agar using the antibiotics (final concentrations in parentheses) ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (5 μg/ml), tetracycline (5 μg/ml), spectinomycin (100 μg/ml), and erythromycin (2 μg/ml) plus lincomycin (25 μg/ml) for macrolide-lincosamide-streptogramin B (MLS) determination. Biofilm assays and growth of cells for microscopy or biochemical analysis were performed with MSgg medium (29). When required, MSgg culture medium was supplemented with 1% threonine. Unless otherwise stated, induced expression was achieved with 1 mM IPTG or 1% (wt/vol) xylose. To generate biofilms, 3 μl of an LB overnight culture was spotted onto 1.5% agar supplemented with 1.5% agar. MSgg plates and incubated for 72 h at 30°C. For liquid cultures, an overnight culture was diluted 1:20 in MSgg medium and grown at 30°C with agitation at 200 rpm until reaching the desired growth stage. If necessary, inducers were added to the culture as stated in the figure legends.

Strain construction. Genomic modifications in B. subtilis were performed according to standard protocols (64). Linearized plasmid DNA or PCR products were brought into cells by inducing natural competence, leading to incorporation of the foreign DNA into the genome by homologous recombination (65). SPP1-mediated phage transduction was used to shuttle constructs among strains, in order to combine mutant alleles (66). For plasmid construction, genes were amplified from genomic DNA with primers carrying a 5’ extension with desired restriction sites to clone the PCR product into the target plasmids. A list of all of the primers used in this study is presented in Table S2 in the supplemental material. For overexpression with the IPTG-inducible Hyperspank promoter, the genes were cloned into pDR111 (67, 68) or into the pBM001 plasmid, a chimera of pDR111 and pDR183 (kindly provided by David Rudner at the Harvard Medical School, Boston, MA). pBM001 has a pDR183 backbone to integrate the plasmid into the lacA locus combined with a fragment from pDR111 that carries the Hyperspank promoter, the multiple-cloning site, and the Lac repressor. The FtsZ-GFP strain was created with the plasmid pX (69), which integrates genes into the amyE locus and expresses them from a xylose-inducible promoter. FtsZ was joined with GFPmut1 by long-flanking homology (LFH) PCR and inserted into pX. For the construction of EzrA-GFP, we made use of the pSG1154 plasmid (70), which allows in-frame fusions to GFPmut1 and further expression under the control of a xylose-inducible promoter. For the independent overexpression of both flotillins and a third xylose-inducible gene, we decided to construct a bicistronic mRNA of floA and floT via LFH-PCR, which was integrated into the lacA locus with pBM001. The strain showed similar overexpression of floA and floT in the presence of IPTG compared to expression of a single gene, as determined by reverse transcription (RT)-PCR (data not shown).

Image analysis. Biofilms were documented using a Nikon SMZ1500 stereo microscope equipped with a Leica DFC295 color camera and Zeiss AxioVision software. Final processing of the images was done with Photoshop. For microscopy, an overnight culture was diluted 1:20 in MSgg medium and grown at 30°C with agitation at 200 rpm until reaching the desired growth stage. If necessary, inducers were added to the culture as stated in the figure legends. Prior to analysis, 1 μM FM4-64 (Invitrogen, Carlsbad, CA) was added to stain the membrane, and Hoechst 33342 was added to a final concentration of 1 μg/ml to stain the DNA. For static images, cells were fixed with 4% paraformaldehyde for 7 min and washed with phosphate-buffered saline (PBS) several times. Finally, cells were spotted on a microscopic slide covered with a 1% agarose pad made with PBS. Images were captured with a Leica DMI6000B microscope equipped with a Leica CFT6000 illumination system coupled with a Leica DFC630FX color camera. Deconvolution was performed with a software algorithm of the LAS AF software. Fluorescence quantification and generation of color spectra were performed with Fiji. Images were processed with Leica LAS AF software and Photoshop.

Flow cytometry. For flow cytometry analysis, 3-day-old biofilms were detached from the agar surface, resuspended in PBS, and mildly sonicated (power output of 0.7 and cycle of 50%) in order to separate cells from extracellular matrix material. Subsequently, cells were fixed with 4% paraformaldehyde for 7 min and washed several times. For analysis, cells were diluted 1:1,000 in PBS. Analysis was carried out with the benchtop MACSquant analyzer (Miltenyi Biotech, Germany). YFP signals were detected.
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00719-13/-/DCSupplemental.

Figure S1, EPS file, 1.9 MB.
Figure S2, EPS file, 1.2 MB.
Figure S3, EPS file, 20 MB.
Figure S4, EPS file, 8.9 MB.
Figure S5, EPS file, 1.2 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.

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