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To Mid-cell and Beyond: Characterizing the Roles of GpsB and YpsA in Cell Division Regulation in Gram-positive Bacteria

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To Mid-cell and Beyond: Characterizing the Roles of GpsB and YpsA in Cell Division

Regulation in Gram-positive Bacteria

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of
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ABSTRACT

The bacterial cell division protein FtsZ is a tubulin homolog that forms a ring-like structure at the site of cell division in most bacterial species. There it acts as a scaffold, aiding in the recruitment of other divisome proteins to the site of cell division. Furthermore, studies focusing on the role of FtsZ treadmilling and septal peptidoglycan synthesis implicates that FtsZ plays a direct role in the ultimate closure of the division septum. Thus, many studies in the field of bacterial cell division have focused on FtsZ in terms of its spatial and temporal regulation as well as its ability to interact with other division proteins. The finding that cells lacking well-studied regulators of FtsZ activity still divide at wild type like capacities suggests the existence of other yet to be discovered factors involved in the cell division process. Work in our lab has focused on the identification and characterization of novel regulators of cell division in the rod-shaped model organism, *Bacillus subtilis*, and in the spherical bacterium, *Staphylococcus aureus*. Here, we describe the function of YpsA, previously annotated as a protein of unknown function, and GpsB in *B. subtilis* and *S. aureus* respectively. Our results indicate that YpsA provides oxidative stress protection in *B. subtilis* cells. Furthermore, YpsA appears to be functioning as a growth rate-dependent cell division inhibitor, though the mechanism by which YpsA elicits this function has not yet been identified. It is interesting to note that this proposed function of YpsA, as well as the growth rate dependency, is also conserved in *S. aureus*. Sequence and structural analysis of YpsA allowed for the identification of several amino acid residues critical for the ability of YpsA to elicit filamentation. We have also found that disruption of a conserved substrate binding groove, through site directed mutagenesis, resulted in an impairment of YpsA-GFP foci formation in addition to cell division inhibition. That had indicated a link between foci
formation and the ability of YpsA to inhibit cell division. A follow up study utilizing a screen to identify intragenic and extragenic suppressors of YpsA-mediated filamentation allowed for the identification of additional amino acid residues important for the function of YpsA, many of which also lined the predicted DNA binding groove. Using this screen, we were also able to isolate an extragenic suppressor mutation in *yfhS*, which was annotated as a sporulation gene based on transcriptomics data. Our results indicate that YfhS may also play a role in cell size regulation during vegetative growth, as cells harboring *yfhS* null mutations appeared smaller in both length and width when compared to a wild type control. Collectively, these results constituted the first reports on the role of YpsA in cell division in *B. subtilis*. Our study focusing on GpsB in *S. aureus* indicated a role for GpsB in regulating the dynamics of FtsZ during the cell cycle. Our results indicate that production of *S. aureus* GpsB in *B. subtilis* is lethal and results in severe filamentation indicative of cell division inhibition. Furthermore, overexpression of *gpsB* in *S. aureus* also resulted in cell division inhibition, and depletion of intracellular GpsB levels resulted in a lethal phenotype thereby confirming its essentiality in *S. aureus*. Fluorescence microscopy revealed that GpsB localization was dynamic during the cell cycle as GpsB-GFP was observed at the leading edge of the invaginating membrane. In combination with *in vitro* analysis of GpsB suggesting a direct interaction with FtsZ, we propose a model where GpsB in *S. aureus* forms lateral interactions between FtsZ protofilaments. This increases the local concentrations of FtsZ at the division site and subsequently triggers FtsZ protofilament disassembly via GpsB activity, and ultimately contributes to FtsZ treadmilling facilitating septum closure.
CHAPTER ONE: INTRODUCTION

Introduction

FtsZ is a Central Protein in Bacterial Cell Division

The ability to reproduce is a fundamental characteristic of life. In bacteria this process is predominately carried out through binary fission, resulting in the production of two similarly sized daughter cells from one parental cell. Much of what is known about cell division in bacteria stems from studies conducted in the rod-shaped model organisms, Gram-negative *Escherichia coli* and Gram-positive *Bacillus subtilis*. Several major factors involved in the division process in these organisms have been previously identified [1, 2]. During cell division in both *E. coli* and *B. subtilis*, these factors localize to mid-cell and form a multiprotein complex termed the divisome, which ultimately facilitates the division event [1, 3, 4]. Divisome assembly, and later constriction to enable septum formation, is highly dependent upon the correct localization and assembly of the tubulin homolog, FtsZ (*filamentation temperature sensitive*), into a ring-like structure at mid-cell termed the Z-ring [5-8].

FtsZ, like eukaryotic tubulin, is a GTPase, and the binding and hydrolysis of guanosine triphosphate (GTP) is essential for its function [2]. The binding of GTP to the active site of FtsZ allows for rapid polymerization of FtsZ monomers existing at critical concentration into tubulin-like protofilaments, which ultimately organize into a ring-like structure at the site of cell division [2, 9, 10]. Disassembly of the Z-ring occurs through the GTPase activity of FtsZ, which is only activated when FtsZ is in a polymeric state. Hydrolysis of GTP into guanosine diphosphate (GDP) results in rapid destabilization of the ring-like structure, and subsequent depolymerization
of FtsZ [2, 11, 12]. Early models had suggested that the hydrolysis of GTP was force generating, resulting in a curved confirmation of FtsZ protofilaments that puts mechanical stress on the bacterial cell membrane [2, 13-16]. In *E. coli*, however, it was discovered that a reduction in the GTPase activity of FtsZ did not alter septum constriction rates, alluding to the possibility that FtsZ and related activity was not likely the primary force generator during septum constriction [17, 18]. The same study also concluded that, based on the relationship between cell elongation and septum constriction, peptidoglycan synthesis was the true rate-limiting step of septum closure [17]. Recent reports support this conclusion and further suggest that GTP-dependent treadmilling of FtsZ filaments within the Z-ring around the site of cell division facilitate peptidoglycan synthesis and insertion into the cell wall at the division septum in decreasingly smaller uniform arcs. Thus, the treadmilling activity of FtsZ can be directly linked to division septum closure [19, 20].

**Division Site Selection in Rod-shaped Model Organisms**

The formation of the Z-ring at future division sites is one of the crucial first steps in the initiation of bacterial cell division [8]. In both *E. coli* and *B. subtilis* cell division occurs at mid-cell, perpendicular to the long axis and along the short axis of the cell. Thus, the production of viable daughter cells is dependent upon the proper localization of FtsZ, and other divisome components, to this mid-cell position [3, 4]. In terms of division site selection, two well-studied spatial regulators of Z-ring positioning have been previously characterized in both *E. coli* and *B. subtilis*, the Min system and nucleoid occlusion [6]. The Min system derives its name from the observation that *E. coli* and *B. subtilis* cells harboring min mutations divide at the cell poles in addition to mid-cell positions, leading to the production of DNA free minicells [6, 21, 22]. In *E. coli*, the Min system is made up MinC, MinD, and MinE proteins, all of which are encoded from
a single operon and all of which have their own distinct functions [6, 23]. MinC is largely considered the effector protein of the Min system, as previous studies have implicated its direct role in the destabilization of FtsZ polymers [24-27].

In *B. subtilis*, the Min system also plays a role in division site selection, functioning in the inhibition of Z-ring assembly in sites that are non-conducive for the production of viable daughter cells [6, 28, 29]. While MinC and MinD are conserved in *B. subtilis*, a MinE homolog is absent in this organism. In addition, MinCD does not oscillate in *B. subtilis* as it does in *E. coli* [6, 30-32]. Another well-conserved cell division protein, DivIVA, was found to be involved in the regulation of the Min system in *B. subtilis* [6, 33, 34]. DivIVA localizes to the cell poles along with MinJ through a direct interaction. MinJ was then believed to recruit MinCD to polar regions leading to the inhibition of Z-ring assembly [35, 36]. Further studies into DivIVA localization had revealed that DivIVA is capable of sensing negative membrane curvature, allowing for its recruitment to the division septum in dividing cells due to the invagination of the cell membrane [37-39]. Additionally, it had been observed that *B. subtilis* MinC also localizes to active division sites along with MinJ, suggesting that that the Min system in *B. subtilis* functions in a manner that is different than that of the Min system in *E. coli* [40, 41]. Furthermore, DivIVA was found to localize to the nascent division sites early on in the division process. Once recruited, DivIVA forms highly stable ring structures on either side of the division septum with MinJ, thus recruiting the MinCD complex to mid cell [42]. Current models of the Min system in *B. subtilis* support the idea that the FtsZ inhibitory complex, MinCD, is sequestered to both sides of the forming divisome at the onset of cell division to prevent the formation of aberrant Z-rings directly next to the newly constructed division septum [40-42].
Another mechanism involved in the spatial regulation of Z-ring assembly is nucleoid occlusion (NO), which prevents the formation of Z-rings over the bacterial chromosome [6, 43]. In *E. coli*, this process is mediated through SlmA, which was identified via a synthetic lethal screen in strains containing a defective Min system. Fluorescence microscopy revealed that SlmA colocalizes with the nucleoid and directly interacts with FtsZ *in vitro* [44]. Further analysis indicated that SlmA interacts with FtsZ through its highly conserved C-terminal domain, which adopts a unique confirmation to facilitate this interaction [45]. In addition, it had been found that overproduction of SlmA results in cell division inhibition, supporting the idea that it is also interacting with the division machinery *in vivo* [44]. In support of this finding, biochemical analysis had revealed that SlmA directly inhibits FtsZ polymerization, an activity that is dependent upon its ability to bind DNA in a sequence specific manner at sites termed SlmA binding sites (SBSs) [46]. Current models suggest that the binding of SlmA to SBSs promotes the disassembly of FtsZ polymers, occluding the nucleoid region as a potential site for Z-ring formation. In addition, in further support of this model, SBSs were found to regulate FtsZ *in vitro*, and cells containing extra copies of SBSs underwent abnormal division in a SlmA dependent manner. These findings had thus identified SlmA as the primary NO effector protein in *E. coli* [43, 46].

Like *E. coli*, *B. subtilis* also has a NO system, however the underlying mechanism of this system differs from that of *E. coli* [43]. In *B. subtilis* NO is mediated through a ParB-like protein, Noc (formerly YyaA). Like ParB, Noc was originally thought to be involved in nucleoid partitioning and organization in *B. subtilis*. In one initial study focusing on the characterization of Noc, immunofluorescence microscopy revealed colocalization between Noc and the nucleoid region. Furthermore, gel mobility shift assays revealed the ability of Noc to bind DNA [47].
Follow-up studies focusing on the nucleoid organization function of Noc had revealed that cells harboring a deletion of noc contained division septa that had formed over the nucleoid region. Overproduction of Noc was also found to result in a block in cell division due to unregulated Z-ring formation. Collectively these data supported the idea that Noc was functioning as a NO system in *B. subtilis* [48]. Later studies had found that Noc binds DNA in a sequence specific manner to regions termed Noc binding sites (NBSs), which are widely distributed across the *B. subtilis* chromosome. NBSs were determined to play an important role in the recruitment of Noc to the nucleoid region. Further analysis into the localization of Noc indicated a close association between it, the cell periphery, and DNA [49]. More recently it was determined that Noc was involved in the recruitment of DNA to the cell membrane, a function that was determined to be essential for Noc to inhibit Z-ring formation. These data support the current model of NO in *B. subtilis* where Noc interacts with the cell membrane and NBSs simultaneously, forming large complexes anchoring the DNA to the cell membrane. It is in these areas where Z-ring formation is physically prevented due to the formation of DNA complexes [50]. Interestingly, it has been found that cell division can occur efficiently in the absence of a functional Min system and NO system in both *E. coli* and *B. subtilis*, indicating the existence of other yet-to-be discovered factors governing Z-ring positioning in these organisms [51, 52].

**Early Divisome Assembly and Positive Regulation of the Z-ring**

In both *E. coli* and *B. subtilis* FtsZ marks the site of cell division, ultimately serving as a scaffold and ensuring the proper localization of other mid-cell associated cell division proteins that form the divisome [4, 6]. In *E. coli*, at least ten different proteins make up the divisome, many of which are conserved in *B. subtilis* [6, 53, 54]. The order in which these proteins are recruited to mid-cell is crucial in maintaining the precision of the cell division process. Amongst
the first proteins to localize to the division site are those that positively regulate Z-ring formation and those that anchor the Z-ring to the bacterial cell membrane, thus beginning the formation of the septal ring [1, 54, 55]. In *E. coli*, the initial stabilization of the Z-ring is accomplished through the early divisome proteins FtsA and ZipA, which have been shown to share overlapping roles as FtsZ membrane anchors [3, 56, 57]. FtsA belongs to the same protein superfamily as eukaryotic actin and other well-defined ATP (adenosine triphosphate) binding proteins [58, 59]. Likewise the binding of ATP by FtsA is critical for its ability to self-interact, and thus is also critical for its ability to form actin-like protofilaments [60, 61]. FtsA self-interaction has been implicated to be essential for proper septation and cell division in *E. coli*, as a deletion of the FtsA 1C domain required for multimerization results in cells that fail to divide normally [62, 63]. Localization studies had revealed that FtsA is recruited to the future site of cell division early on in a FtsZ dependent manner [64]. FtsA was found to interact directly with the highly conserved C-terminal tail region (CTT) of FtsZ, utilizing the same resides as ZipA [65-67].

Studies focusing on *B. subtilis* FtsA have also revealed a direct interaction between FtsA and FtsZ in this organism. Additionally, it has been suggested that FtsA associates with FtsZ prior to the formation of the Z-ring, suggesting that FtsA colocalizes with FtsZ to mid-cell and helps maintain the efficiency of Z-ring formation during the early steps of cell division [68]. Interestingly, another protein, SepF (YlmF), has been found to have some overlapping roles with FtsA in *B. subtilis* [6]. Biochemical analysis has indicated that SepF is able to assemble into large ring-like structures that are essential for its ability to promote FtsZ polymerization and protofilament bundling [69]. SepF was found to interact directly with FtsZ’s C-terminus in a manner similar to FtsA [70]. Furthermore, SepF also contains a conserved membrane tethering sequence (MTS) allowing for SepF to anchor bound FtsZ to the bacterial cell membrane [3, 70,
SepF is considered to be non-essential in *B. subtilis*, however cells lacking *sepF* display a moderate cell division phenotype. Deletion of the *ylmB-H* operon, in which *sepF* is encoded, was observed to result in elongated cells when compared to wild-type. It is important to note that these cells still contain Z-rings, although the space between Z-rings is greater than that of wild-type cells, corresponding to the increase in cell length [72]. Mutations in *sepF* have been found to become synthetically lethal in cells harboring deletions in either *ftsA* or in *ezrA*, a gene that encodes another cell division protein indicated to negatively regulate Z-ring assembly [71, 73].

Other well-conserved positive regulators of Z-ring formation have also been characterized in both *E. coli* and *B. subtilis*. Many of these regulators function in the lateral bundling of FtsZ protofilaments during Z-ring formation, and in ensuring the overall stability of the Z-ring during the division process [3]. ZapA (Z-ring associated protein A) is one such protein involved in Z-ring stability that is recruited to the site of cell division early on in a FtsZ-dependent manner [2, 74]. ZapA was initially discovered in *B. subtilis* however at least 35 confirmed homologs have been identified in various species of bacteria including *E. coli* [74]. Although dispensable, ZapA is considered to play a direct role in the division process in both *B. subtilis* and *E. coli* [2]. In *B. subtilis*, ZapA localizes to mid-cell, and biochemical analysis has revealed a direct interaction between ZapA and FtsZ. Furthermore, light scattering assays have indicated that ZapA promotes the polymerization of FtsZ, indicating a potential function in the early development of the Z-ring. It is also interesting to note that, like *sepF*, mutations in *zapA* are synthetically lethal in *B. subtilis* cells also harboring a knockout in *ezrA*. Additionally, cells lacking *zapA* are sensitive to reduced intracellular FtsZ concentrations, furthering the argument that ZapA is a component of the early cell division machinery [74]. Likewise, studies in *E. coli*
have also indicated a role for the ZapA ortholog, YgfE, in FtsZ polymerization and in polymer bundling, indicating a highly conserved role between ZapA and YgfE in *B. subtilis* and *E. coli* respectively [2, 74, 75].

**Negative Regulation of the Z-ring and Ties to Other Essential Cellular Functions**

In addition to the Min system and NO, which have been identified as regulators of Z-ring positioning that spatially govern where Z-rings may form, other negative regulators of Z-ring assembly have been identified in both *E. coli* and *B. subtilis*. These regulators further facilitate the accuracy of Z-ring formation and, in addition, some can prevent further cell division events if conditions do not favor the production of viable daughter cells [2, 3, 76]. EzrA is one such divisome associated protein that was identified in *B. subtilis* and is highly conserved in Gram-positive bacteria [54]. Initial studies focusing on characterizing EzrA in *B. subtilis* observed an increase in Z-ring formation in cells harboring an *ezrA* knockout, particularly at the cell poles. Furthermore, the critical concentration of FtsZ required for Z-ring formation was also significantly lower in a *ezrA* null strain than in wild type cells, supporting the argument that EzrA was acting as a negative regulator of FtsZ polymerization [77]. EzrA was found to localize to both the bacterial cell membrane and to the divisome of dividing *B. subtilis* cells. Divisome localization was found to be FtsZ dependent, and a direct interaction between EzrA and FtsZ has been observed *in vitro* [77, 78]. Overexpression of *ezrA* resulted in impaired Z-ring assembly and filamentation, indicating that cell division was being inhibited. Light scattering assays later confirmed the suspected ability of EzrA to inhibit FtsZ polymerization [78].

Other inhibitors of Z-ring assembly are induced and function by responding to various signals during the bacterial cell cycle [2]. Previous work in *B. subtilis*, for example, has indicated that growth rate influences the frequency of Z-ring formation. Cells grown in minimal
medium were found to have more infrequent Z-rings formed than wild type cells grown in nutrient rich medium at the same growth temperature. Interestingly, it was also found that the overall intracellular FtsZ concentration remains the same regardless of growth rate and in addition, even cells containing an extra copy of ftsZ were unable to overcome this growth rate-dependency [79]. Further studies revealed that a constant relationship between cell length and Z-ring formation was maintained regardless of growth rate, indicating that cells must reach critical mass prior to a division event [80]. The ability of B. subtilis cells to time division events with cellular growth indicated that cells must not only be able to sense their own growth rate and factors such as available nutrients, but also utilize this information as a signal to the appropriate cell division regulator(s) in order to alter division rates [81]. Further work identified UDP-glucose, which plays a vital role in the production of teichoic acids in Gram-positive bacteria, as a potential signaling molecule in this pathway as mutations in enzymes responsible for its production (pgcA and gtaB) resulted in significantly shorter cells when compared to a wild-type control [80-82]. In addition, similar findings in E. coli had revealed that mutations in the pgcA homolog, pgm, also resulted in a truncated cell length, indicating that this pathway and the function of UDP-glucose may be conserved across multiple species [81, 83]. UgtP, a non-essential protein whose enzymatic activity functions in converting UDP-glucose into teichoic acid, was later implicated to play a role in regulating cell division in B. subtilis in relation to growth rate [84]. Cells lacking ugp (formerly ypfP) were observed to be significantly shorter than wild type cells when grown in nutrient rich medium, however no difference was observed when cells were grown in minimal medium [81, 85]. Additionally, fluorescent protein tagged UgtP was observed to localize to the cell poles in addition to the division septum in dividing B. subtilis cells, suggesting a potential interaction with the cell division machinery. Mid-cell
 localization of UgtP was found to be more frequent in rapidly growing cells grown in nutrient rich medium [80, 81, 84]. Further experimentation revealed that purified UgtP is able to inhibit FtsZ polymerization in vitro, and the addition of external UDP-glucose promoted this activity indicating that UgtP was potentially responsible for the inhibition of Z-ring assembly in nutrient rich conditions [80, 84]. Interestingly, the overall level of UgtP was also determined to be dependent on nutrient availability. When grown in minimal media, there was an approximate 6-fold decrease in UgtP levels when compared to cells grown in nutrient rich medium. Collectively these data support a model where UgtP directly inhibits Z-ring assembly in rapidly growing cells (in nutrient rich conditions), allowing cells to reach a critical mass prior to dividing, preventing premature division events. In addition, this delay in Z-ring formation also allows for proper chromosome segregation in dividing cells [80].

Cellular stress, such as DNA damage, can also result in the inhibition of the cell division machinery in both E. coli and B. subtilis, although the mechanism is much better understood in E. coli via SulA [2, 3]. Original studies focusing on SulA (formerly SfiA) and SulB (formerly SfiB) in E. coli had coupled these proteins to the SOS-response, as mutations in sulA and sulB were found to restore wild-type like cell division in strains where the SOS-response would have resulted in a division block [86]. Furthermore, follow-up analysis had indicated that overexpression of sulA also results in cell division inhibition and associated filamentation in E. coli. This had further suggested that SulA was acting as a cell division antagonist facilitating SOS-associated cell division inhibition in this organism [26, 87]. SulA was found to interreact directly with FtsZ preventing its polymerization, ultimately resulting in a block in septation and division [88, 89]. More recent studies had proposed a model where SulA acts in a manner that sequesters FtsZ monomers, preventing adequate FtsZ concentrations required for Z-ring
formation [90]. Studies focusing on the stability of SulA had revealed that following its production in response to DNA damaging conditions, SulA is rapidly degraded by the Lon protease, allowing for the resumption of normal cell division following DNA repair [2, 91-93].

In B. subtilis, SOS mediated cell division inhibition is thought to occur through YneA. Although YneA does not share significant sequence homology with SulA from E. coli, it does appear to be regulated in a similar manner [94]. YneA was found to contain three potential LexA (DinR) binding sites within its promoter region [94, 95]. Furthermore, UV radiation and the DNA damaging agent, mitomycin C (MMC), were found to induce the expression of yneA and other SOS genes within the same regulon in a RecA dependent manner [95]. Further elucidation of the yneA operon (comprised of genes yneA, yneB, and ynzC) confirmed the role of LexA in its transcriptional regulation, as a deletion in lexA results in an upregulation of these genes. Furthermore, deletion of the yneA operon is able to suppress filamentation that was observed resulting directly from a lexA mutant, suggesting that at least one of these genes encodes a potential SOS associated cell division inhibitor. Additional experimentation revealed that ectopic expression of yneA alone resulted in filamentation, and thus was enough to inhibit cell division [96]. While a direct interaction between YneA and FtsZ has not been confirmed yet, filamentation resulting from yneA overexpression was determined to be due to a delay in Z-ring assembly. Furthermore, yneA overexpression also results in a defect in daughter cell separation in cells that are able to form division septa [95, 97]. It is important to note that cell division is able to continue normally, at wild-type like capacity, when yneA is no longer being overexpressed following the removal of inducer [97]. The specific mechanism by which YneA acts to inhibit cell division has not yet been elucidated, however the full length of the protein does appear to be required to carry out this function [94]. YneA contains both an essential
transmembrane region and a C-terminal LysM domain, which is cleaved following the SOS response allowing for rapid proteolytic degradation of YneA. This in turn allows for the relief of SOS associated cell division inhibition immediately following the response [94, 97].

**Cell Elongation and the Maintenance of Cell Shape**

Cell division and elongation are intrinsically linked though the process of peptidoglycan (PG) synthesis, which occurs either at mid cell along the short axis during division events or along the long axis of rod-shaped bacteria during elongation [98, 99]. Made up of glycan strands bridged together by peptide cross-links, PG is a major structural component of the cell wall in many bacterial species as it forms a protective layer exterior to the cell membrane and functions in maintenance of cell shape [100, 101]. Synthesis of PG is a multi-step process that is catalyzed by PG synthases that either function in the polymerization of glycan strands, glycosyltransferases, and/or in the formation of the peptide cross-links, transpeptidases. These synthases can either be bifunctional, such as the class A penicillin binding proteins (PBPs), or monofunctional transpeptidases (class B PBPs) or glycosyltransferases [99]. It is interesting to note that many of the PBPs interact with both the cell division and elongation machinery, providing a direct link between the two processes [98].

In both *E. coli* and *B. subtilis* cellular growth (elongation) and cell shape maintenance are regulated via the conserved actin-like protein, MreB [102, 103]. Initially characterized in *E. coli*, mutations in *mre* genes were observed to result in distinct morphological defects [104]. Further work in *B. subtilis* revealed that, unlike in *E. coli*, *mreB* is essential and required for cell viability [32, 105, 106]. Depletion of intracellular MreB levels, however, resulted in significant changes in cell shape such as cell rounding and bulging. Furthermore, immunofluorescence microscopy used to examine MreB localization had indicated the formation of helical filaments beneath the
cell membrane near mid-cell [105]. It is interesting to note that *B. subtilis* also contains two other MreB-like proteins, Mbl and MreBH [103]. Cells harboring mutations in *mbl* also display defects such as bulging and rounding, as well as cell bending. Furthermore, *mbl* was also observed to form helical filaments, similar to MreB, that extend the full length of the cell [105]. Mutations in *mreBH* resulted in a narrow cell phenotype, and MreBH localization studies revealed similar helical patterns to Mbl. Further experimentation revealed that MreB, Mbl, and MreBH each colocalize within *B. subtilis* and play partially redundant roles in governing cell shape and width [107, 108]. Interestingly, MreB and Mbl were each found to play pivotal roles in the synthesis of PG within the lateral cell wall. Phenotypes associated with MreB depletion and mutations within *mbl* were therefore attributed to this defect in PG synthesis [107].

During the cell cycle MreB functions in a multiprotein complex termed the elongasome (also known as the elongase). Other proteins within this complex in *E. coli* include those that are essential for cell elongation (MreC and MreD), cell shape determination (RodA), PBPs, and other membrane bound proteins that are involved in the processing of PG precursors (MurG and MraY) [109]. Work in *B. subtilis* later indicated potential interactions between MreB with MreC, MreD, RodA, and various PBPs within the elongasome [103]. Mechanistically, MreB is thought to function in the spatial regulation of these cell wall remodeling factors in areas where wall expansion is required, thus acting as a scaffolding protein in a manner similar to FtsZ [103, 105, 110, 111]. Furthermore, additional MreB interaction partners have been identified, most of which are involved in cell wall synthesis, outside of those proteins that primarily make up the elongasome [103, 109, 112]. Interestingly, in *E. coli*, MreB was found to directly interact with FtsZ. Through time-lapse fluorescence microscopy, MreB was found to be recruited to the divisome early on in a FtsZ-dependent manner. Additional work had indicated that the amino
acid residue MreB\textsuperscript{D285} was critical in mediating the interaction between these two scaffolding proteins. In addition to disrupting MreB-FtsZ interactions, mutations in this residue also affected the localization of PBPs required for proper septal synthesis during cell division, thus providing a direct connection between elongasome and divisome functioning [113]. In addition, current work supports the hypothesis that the elongasome developed evolutionarily from the divisome. Evidence to support this includes shared PBPs functioning in both cell elongation and septal synthesis, interaction of both protein complexes with PG precursors, and similar actin-like filaments that exist in both the elongasome and divisome [98, 114]. Work in \textit{E. coli} further supports the idea that both the elongasome and divisome utilize the same PBPs in order to drive PG synthesis at proper locations during the cell cycle. Specifically, PBP2 was found to be required for both MreB-directed and FtsZ-directed PG synthesis [115]. More interestingly, evidence has suggested that the role of FtsZ in PG synthesis could compensate for MreB in cells where MreB levels were depleted [115, 116]. Additional work focusing on the overlap of PBPs between cell elongation and division indicated colocalization between PBP2 and PBP3 at mid-cell during division events. These PBPs were determined to interact and, in addition, PBP2 was determined to directly interact with some divisome components including PBP3 FtsQ, FtsW, and FtsN [114].

Coordination between the cell wall remodeling and the cell division machinery in \textit{B. subtilis} has also been extensively investigated. PBPs play an integral role in facilitating this link by directing the synthesis of cell wall precursors in regions critical for the formation of the division septum. More specifically, PBP2B has been implicated in having a direct role in the division process in \textit{B. subtilis} [117-119]. Reduction of intracellular levels of PBP2B was observed to result in filamentation characteristic of cell division inhibition, and in the improper
localization of some late stage divisome factors, but not FtsZ. In terms of division septa formation, depleted levels of PBP2B resulted in significantly reduced invagination and in visual phenotypic abnormalities [120]. It is important to note that later work had determined that PBP3, normally not required for cell viability, becomes essential in cells containing depleted levels of PBP2B. This had indicated some functional overlap between the two PBPs [121].

PBP1 (ponA) has also been implicated as an important factor in both cell elongation and cell division in B. subtilis [122-124]. In terms of cell division, PBP1 localizes to division sites in a manner that is dependent on other cell division proteins, including FtsZ and PBP2B. Furthermore, PBP1 was determined to play an important role in B. subtilis sporulation, functioning in the development of the asymmetric division septum [123]. Additional work focusing on the localization of PBPs in B. subtilis using fluorescence microscopy had indicated that, in addition to septal localization, PBP1 was retained at the cell poles in some cases and weak fluorescence signal was also observed around the periphery of the cell [125]. Interestingly, PBP1 was also found to localize to the lateral cell wall in non-dividing cells, suggesting additional roles of this PBP in the cell elongation machinery. These various localization patterns of PBP1 had suggested its ability to dynamically switch between playing a role in cell elongation and in cell division [122]. This switch was later determined to be influenced by the late divisome protein, GpsB (formerly YpsB) [103, 122]. GpsB (guiding PBP1 shuttling) is paralogous to DivIVA and was found to localize to the division site in B. subtilis [126]. This localization pattern was determined to be dependent on other later stage cell division proteins, and thus further indicated the role of GpsB in the divisome complex [126, 127]. Interestingly, further work had determined that cells lacking both gpsB and ezrA (which encodes a known cell division inhibitor as discussed above) results in a sick phenotype not observed in single knockout
strains. This sick phenotype was attributed to defects in cell wall synthesis at both polar locations and at newly formed division septa [122]. Furthermore, in *B. subtilis* was determined to interact with both the elongation and cell division machinery, guiding the localization of PBP1 from the lateral cell wall to division sites during division cycles [103, 122]. A list of key cell division related proteins in *B. subtilis* is outlined in Table 1.

**Cell Division in Non-model Organisms: *Staphylococcus aureus***

While traditional cell division studies have focused on the rod-shaped model organisms *E. coli* and *B. subtilis*, a recent shift in interest has resulted in an influx of work on non-model systems. This includes studies that have suggested alternative mechanisms governing the spatial and temporal regulation of the division process [128]. In the Gram-positive opportunistic pathogen *Staphylococcus aureus*, division takes place sequentially along three orthogonal division planes. Ultimately, this style of cell division is what yields the grape-like cell morphology where the name *Staphylococcus* is derived [128-130]. Unlike *E. coli* and *B. subtilis*, where division site selection is based upon localization of divisome components to mid-cell, cell division in *S. aureus* can occur at any equatorial plane. How the division site is selected, however, still remains an open question [131]. This is further complicated by the fact that *S. aureus* lacks a Min system, yet still contains DivIVA that was shown to regulate Min system localization in *B. subtilis* [42, 131]. *S. aureus* is known to have NO system however, when compared to NO systems in *E. coli* and *B. subtilis*, relatively little is known [128]. *S. aureus* cells harboring a mutated copy of the gene that encodes the NO protein were observed to have an increased cell diameter compared to wild type, and division septa were observed over the chromosome leading to DNA breaks. Fluorescence microscopy examining a *fisZ-cfp* containing strain revealed abnormal Z-ring formation in mutant cells. Interestingly, the use of a DNA
replication inhibitor also resulted in aberrant Z-ring formation, supporting a hypothesis that chromosome dynamics, and therefore NO, contribute to division site selection in *S. aureus* [132].

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>FtsZ</td>
<td>Core divisome component, acts as a scaffold in the recruitment of divisome proteins to site of cell division.</td>
</tr>
<tr>
<td>MinC</td>
<td>Min system component, inhibition of Z-ring assembly, septum placement</td>
</tr>
<tr>
<td>MinD</td>
<td>Min system component, inhibition of Z-ring assembly, septum placement</td>
</tr>
<tr>
<td>Noc</td>
<td>Nucleoid occlusion</td>
</tr>
<tr>
<td>DivIVA</td>
<td>Divisome protein, septum placement via interaction with the Min system</td>
</tr>
<tr>
<td>FtsA</td>
<td>FtsZ membrane anchor, divisome component</td>
</tr>
<tr>
<td>SepF</td>
<td>Divisome component, alternative FtsZ membrane anchor</td>
</tr>
<tr>
<td>ZapA</td>
<td>Divisome component, positive regulator of Z-ring assembly</td>
</tr>
<tr>
<td>EzrA</td>
<td>Divisome component, negative regulator of Z-ring assembly, prevents aberrant Z-rings</td>
</tr>
<tr>
<td>UgpP</td>
<td>Inhibitor of Z-ring assembly, functions in production of glucolipids</td>
</tr>
<tr>
<td>YneA</td>
<td>SOS response protein, cell division inhibitor</td>
</tr>
<tr>
<td>MreB</td>
<td>Core component of the elongasome, functions in cell shape maintenance</td>
</tr>
<tr>
<td>Mbl</td>
<td>MreB-like, functions in cell shape maintenance</td>
</tr>
<tr>
<td>MreBH</td>
<td>Cell shape determining protein</td>
</tr>
<tr>
<td>PBP1</td>
<td>Glycosyltransferase and transpeptidase, functions in both cell division and elongation</td>
</tr>
<tr>
<td>GpsB</td>
<td>Divisome component, regulator of PBP1 localization from lateral cell wall during elongation to the division septum during cell division</td>
</tr>
<tr>
<td>YpsA</td>
<td>Growth rate-dependent cell division inhibitor (this study)</td>
</tr>
</tbody>
</table>

Another conserved protein thought to contribute to division site selection as well as cell size regulation in *S. aureus* is EzrA [131]. Depletion of intracellular EzrA levels was observed to result in cell size variation and was also observed to affect the localization of other divisome components including GpsB and PBP2 [133]. Additionally, EzrA was determined to colocalize with FtsZ at the division site in *S. aureus* [133, 134]. Further work later confirmed that these two division proteins directly interact in this organism [135]. In terms of cell shape regulation, EzrA was determined to play a role in regulating PG synthesis at the division septum by influencing the proper localization of PBPs, particularly PBP2 [134]. It is interesting to note that
PG synthesis and cell wall remodeling have been implicated to aid in division site selection in *S. aureus* [131].

**Project Aims**

The overarching goal of this dissertation is to further our understanding of cell division regulation in the Gram-positive organisms *B. subtilis* and *S. aureus*. The aim of chapters 2 and 3 is to elucidate the function of YpsA, a previously uncharacterized protein highly conserved within the Firmicutes phylum. In chapter 2 we utilize a variety of techniques, including classical bacterial genetics and high-resolution fluorescence microscopy, to examine the role that YpsA plays in regulating cell division in *B. subtilis*. In addition to examining the effects of *ypsA* null mutations and *ypsA* overexpression, we also use structural and sequence information to examine conserved amino acid residues predicted to be critical for the structure and/or function of YpsA. Furthermore, we also examine the role of YpsA in *S. aureus* to determine if the function of YpsA is broadly conserved in the Firmicutes. Chapter 3 of this dissertation is a follow up study of chapter 2 where we use a spontaneous suppressor screen to exploit the lethal YpsA overproduction phenotype. In doing so, we are able to isolate both intragenic and extragenic suppressor mutations that prevent YpsA-mediated lethality in *B. subtilis*. Follow up analysis of the intragenic mutations identifies critical amino acid residues important for the structure and/or function of YpsA. Examination of extragenic suppressor mutations sheds light on a potential mechanism by which YpsA is functioning.

In Chapter 4 we discuss the highly conserved cell division protein, GpsB, in *S. aureus*. The aim of this chapter is to elucidate the novel function of GpsB in regulating FtsZ dynamics in this organism. In addition to examining GpsB overproduction and depletion phenotypes, we also utilize various microscopy techniques, including structured illumination microscopy, to examine
the localization patterns of GpsB in *S. aureus* throughout the cell cycle. Based on previous *in vitro* studies that have identified a direct interaction between GpsB and FtsZ [136], we examine how GpsB influences divisome assembly, and alternatively how divisome assembly affects GpsB localization, through fluorescence microscopy.
CHAPTER 2: DECIPHERING THE ROLE OF A SLOG SUPERFAMILY PROTEIN

YpsA IN GRAM-POSITIVE BACTERIA

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CHAPTER 3: SUPPRESSORS OF YpsA-MEDIATED CELL DIVISION INHIBITION

IN *Bacillus subtilis*

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CHAPTER 4: GpsB IS ESSENTIAL IN REGULATING FtsZ DYNAMICS IN

*Staphylococcus aureus* CELL DIVISION

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Abstract

Bacterial cell division is an essential process that is under the control of multiple different regulatory mechanisms that ensure the production of viable daughter cells. Most of these mechanisms involve a direct or indirect interaction with FtsZ, a tubulin homolog that forms a ring structure (Z-ring) at the site of cell division in most bacteria and functions as a scaffolding protein for the recruitment of additional divisome proteins. Most of what we know about how cell division is regulated stems from studies conducted in the rod-shaped model organisms *Escherichia coli* and *Bacillus subtilis*, both of which divide by constructing a division septum that runs perpendicular to the long axis of the cells. Cell division regulation in some non-model organisms, such as *Staphylococcus aureus*, which switches between division planes orthogonally, is much less well understood. This is in part due to this bacterium lacking several well conserved cell division regulatory systems found in other species. In this study we characterize the role of an essential cell division protein, GpsB, in *S. aureus*. Although highly conserved across the Firmicutes phylum of bacteria, the GpsB ortholog in *S. aureus* appears to
have adapted an alternative role in cell division as it appears to directly influence FtsZ localization and divisome formation. Additionally, GpsB localizes to division sites in cells that are actively undergoing cell division where it is present at the leading edge of the invaginating membrane. These observations and the results of in vitro studies support a model in which GpsB stimulates lateral interactions between FtsZ protofilaments at the site of cell division, and ultimately facilitates FtsZ constriction and treadmilling through stimulating the GTPase activity of FtsZ.

**Introduction**

Studies in bacterial cell division have largely focused on rod-shaped model organisms, *E. coli* and *B. subtilis*, in order to gain a fundamental understanding of the various mechanisms that facilitate this process [2, 3, 8]. While both of these species share a significant amount in common in terms of the make-up of the divisome as well as the spatial and temporal regulation of its formation, a significant knowledge gap exists in the understanding cell division regulation in non-model systems [128]. The Gram-positive opportunistic pathogen, *Staphylococcus aureus*, is one organism in which there has been a recent increase in fundamental biological studies [131]. This is, in part, due to a rapid rise in antibiotic resistance in this organism. Many of these studies have been focused on understanding the mechanisms underpinning divisome localization and assembly, as well as cytokinesis, and several labs have begun work on identifying components of the bacterial divisome as novel therapeutic targets [131, 139-141].

Unlike *E. coli* and *B. subtilis*, which divide along a single division plane, the spherical organism, *S. aureus*, divides sequentially along orthogonal division planes that ultimately result in the formation of its characteristic cell morphology [2, 8, 128, 131]. Despite increases in work focusing on cell division regulation in this organism, the way in which future division sites are
selected as well as how essential divisome proteins localize correctly is poorly understood. This question is increasingly complicated by the fact that S. aureus lacks several well characterized cell division regulators found in rod-shaped organisms. Among those division related proteins that are found within S. aureus is GpsB, a late stage division protein that is paralogous to the well-characterized division protein, DivIVA [131].

GpsB is highly conserved within the Firmicutes phylum of bacteria, and initial characterization was completed in B. subtilis [126]. In B. subtilis, GpsB functions in regulating the dynamic localization of PBP1 between the cell poles and new division sites through a direct interaction. Further analysis has revealed division site localization of GpsB as well as a direct interaction with the negative Z-ring regulator, EzrA [122, 126]. Although non-essential in B. subtilis, cells lacking gpsB were found to have a synthetic lethal phenotype when combined with an ezrA mutation that included defects in the formation of the division septa. Due to the overlapping roles of PBP1 in both cell division and elongation in B. subtilis, GpsB was suspected to play a role in mediating the switch between the two cellular processes [122, 127]. GpsB was also found to interact with class A PBPs in both Streptococcus pneumoniae and Listeria monocytogenes, where the primary function also appears to be conserved [126, 142-144]. Although also conserved in S. aureus, little is known about the function of GpsB in this organism, however it is considered an essential protein [126, 145].

In this study, we characterize the role of GpsB in regulating cell division in S. aureus. We have shown that production of S. aureus GpsB in a non-native host, B. subtilis, is lethal, and results in severe filamentation that is caused by an inhibition in Z-ring assembly. Furthermore, overexpression of S. aureus gpsB in its native host results in cell enlargement that is also consistent with cell division inhibition in this organism. Consistent with previous findings, we
find that gpsB is essential in S. aureus and that depletion of intracellular GpsB levels through the production of gpsB antisense RNA also results in a significant growth defect when compared to a vector control. GpsB localizes to the division site in actively dividing S. aureus cells, and structured illumination microscopy (SIM) revealed that it is present at the leading edge of the invaginating membrane. Furthermore, GpsB and FtsZ were determined to influence each other’s mid-cell localization. Our results in combination with in vitro studies confirm a direction interaction between GpsB and FtsZ. This promotes FtsZ bundling and stimulates the GTPase activity of FtsZ, which supports a model in which GpsB binds FtsZ and promotes lateral interactions between protofilaments. These interactions thereby increase the concentration of FtsZ resulting in increased GTP hydrolysis and ultimately Z-ring disassembly.

**Materials and Methods**

**Strain Construction**

All B. subtilis strains used during the course of this study are derivatives of the laboratory strain, PY79 [146]. Furthermore, all S. aureus strains are derivatives of SH1000 [147]. Table 2 contains all relevant strain information, and Table 3 contains all relevant oligonucleotide information. Production of S. aureus GpsB (GpsB<sub>Sa</sub>) in B. subtilis was achieved by PCR amplifying gpsB<sub>Sa</sub> with primers oP36/oP38 (HindIII/SphI) and cloning the resulting PCR product into the 5’ HindIII and 3’ SphI restriction sites of pDR111 (D. Rudner), placing gpsB under the control of the isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible P<sub>hyperspank</sub> promoter. This plasmid was named pGG3. Production of GpsB<sub>Sa</sub>-GFP in B. subtilis was achieved by PCR amplifying gpsB<sub>Sa</sub> and gfp using the primers oP36/oP37 (HindIII/Nhel) and oP46/oP24 (Nhel/SphI) respectively. The resulting PCR products were ligated together and cloned into the 5’ HindIII and 3’ SphI restriction sites of pDR111 resulting in plasmid pGG4. The engineered
plasmids were then integrated into the non-essential *amyE* locus of the *B. subtilis* chromosome via double crossover homologous recombination. Likewise, overproduction of *B. subtilis* GpsB (GpsB^{Bs}) was achieved using similar methodology. *gpsB^{Bs}* was PCR amplified using primers oP100/oP102 (SalI/NheI), and the resulting PCR product was cloned into the 5’ Sall and 3’ Nhel restriction sites of pDR111. The resulting plasmid was named pGG5. Overproduction of GpsB^{Bs}-GFP was achieved by PCR amplifying *gpsB^{Bs}* using primers oP100/oP101 (SalI/NheI) and *gfp* using primers oP46/oP24 (Nhel/SphI). The resulting PCR products were ligated together and cloned into the 5’ Sall and 3’ SphI restriction sites of pDR111 resulting in the production of plasmid pGG6. Production of GpsB^{Sa} in *S. aureus* was achieved by PCR amplifying *gpsB^{Sa}* using primers oP36/oP38 (HindIII/SphI) and cloning the resulting PCR fragment into the 5’ HindIII and 3’ SphI restriction sites of the pCL15 plasmid [148], placing the gene of interest under the control of the IPTG inducible *P_{spac}* promoter and resulting in plasmid pPE45. To construct a GFP tagged variant of GpsB^{Sa}, *gpsB^{Sa}* and *gfp* were PCR amplified using primer pairs oP36/oP37 (HindIII/Nhel) and oP46/oP24 (Nhel/SphI) respectively. The resulting PCR fragments were ligated together and cloned into the 5’ HindIII and 3’ SphI restriction sites of pCL15, resulting in plasmid pPE46. Production of the GpsB^{Bs} ortholog in *S. aureus* was achieved by PCR amplifying *gpsB^{Bs}* with primers oP100/oP195 (SalI/BamHI). The resulting PCR fragment was cloned into the 5’ Sall and 3’ BamHI restriction sites of pCL15, resulting in plasmid pPE83. In order to construct a strain capable of producing *gpsB^{Sa}* antisense RNA and ribosome binding site under the control of a xylose-inducible promoter, PCR was completed using the primer pair oP187/oP188 (EcoRI/BamHI), and the resulting PCR product was cloned into the 5’ EcoRI and 3’ BamHI restriction sites of the plasmid pEPSA5 [149]. The resulting plasmid was named pGG59. Expression of *zapA-gfp* in *S. aureus* was achieved by PCR
amplifying \textit{zapA} and \textit{gfp} with primers oP236/oP237 (SalI/NheI) and oP46/oP47 (NheI/BamHI) respectively. The resulting PCR products were ligated together and cloned into the 5’ SalI and 3’ BamHI restriction sites of pJB67 [150], placing the gene of interest under the control of a cadmium inducible promoter. The resulting plasmid was named pRB42. Engineered plasmids were first introduced into \textit{S. aureus} RN4220 via electroporation, followed by being transduced into SH1000.

\textbf{Table 2: Strains Used in This Study}

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{B. subtilis}</td>
<td>PY79</td>
<td>Wild type</td>
<td>[146]</td>
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<tr>
<td>\textit{B. subtilis}</td>
<td>GG7</td>
<td>\textit{amyE}:\textit{P}_{\text{hyperspank}}-\textit{gpsB}^{\text{se}} \textit{spec}</td>
<td>This study</td>
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<td>\textit{amyE}:\textit{P}_{\text{hyperspank}}-\textit{gpsB}^{\text{se}}-\textit{gfp}</td>
<td>This study</td>
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<td>\textit{B. subtilis}</td>
<td>GG35</td>
<td>\textit{ΔezrA}:\textit{spec}::\textit{erm} \textit{amyE}:\textit{P}_{\text{hyperspank}}-\textit{gpsB}^{\text{se}} \textit{spec}</td>
<td>Derived from FG345, [74]</td>
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<td>\textit{B. subtilis}</td>
<td>CS26</td>
<td>\textit{ΔponA}:\textit{erm} \textit{amyE}:\textit{P}_{\text{hyperspank}}-\textit{gpsB}^{\text{se}} \textit{spec}</td>
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<td>Derived from BKE15770, BGSC*</td>
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<td>This study</td>
</tr>
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<td>Derived from KR546, [151]</td>
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<td>\textit{B. subtilis}</td>
<td>GG9</td>
<td>\textit{amyE}:\textit{P}_{\text{hyperspank}}-\textit{gpsB}^{\text{se}} \textit{spec} \textit{fixAZ} \textit{fixAZ}-\textit{gfp} \textit{erm}</td>
<td>This study</td>
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<td>SH1000; \textit{pGG59} (\textit{pEPSA5} backbone, \textit{P}_{\text{spa}}-\textit{gpsB}^{\text{se}} \textit{antisense} \textit{bla} \textit{cat})</td>
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*BGSC: Bacillus Genetic Stock Center

\textbf{Media and Culture Conditions}

For \textit{B. subtilis}, cultures were grown at 22 °C overnight in Luria-Bertani growth medium (LB) and subsequently back diluted 1:20 into fresh LB medium. Cultures were then grown to mid-logarithmic phase (OD$_{600}$=0.5) at 37 °C while shaking, and 1 mM IPTG was added (where
required to induce the expression of gene(s) of interest) for three hours unless otherwise noted. For *S. aureus*, cultures were grown at 22 °C overnight in tryptic soy broth (TSB) supplemented with 15 µg/ml chloramphenicol or 5 µg/ml erythromycin where required for plasmid maintenance. On the following day cultures were diluted 1:20 into fresh TSB containing appropriate antibiotics and grown to mid-logarithmic phase. Expression of genes or antisense RNA of interest was induced by the addition of 1 mM IPTG, 1% xylose, or 1.25 µM CdCl₂, where required, directly to the growth medium. FtsZ inhibition experiments were completed by growing the appropriate strains to mid-logarithmic phase and then adding 2 µg/ml of PC190723 (described previously) directly to the culture medium [152]. Samples were harvested after 3 hours.

**Table 3: Oligonucleotides Used in This Study**

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Restriction sites are underlined, and ribosome binding sites and stop codons are in italics.

**Microscopy**

All fluorescence microscopy used during the course of this study was completed as described previously [153]. Briefly, 1 ml aliquots of *B. subtilis* or *S. aureus* cultures to be imaged were washed in phosphate buffered saline (PBS), and bacterial cell pellets were then resuspended in 100 µl of PBS containing 1 µg/ml of the fluorescent membrane stain FM6-64.
and/or 2 µg/ml DAPI in order to visualize the bacterial cell membranes and DNA respectively. Following resuspension, 5 µl aliquots of prepared sample was spotted onto glass bottom dishes (MatTek) and the sample was covered with a 1% agarose pad made with sterile water. Still imaging was completed at room temperature. For time-lapse imaging of SH1000 pGG59 cells, cells were grown in TSB containing 15 µg/ml chloramphenicol (for plasmid maintenance) until an OD$_{600}$=0.5 and 5 µl of culture was then spotted onto a glass bottom dish. The culture was then covered with a 1% agarose pad made with TSB/chloramphenicol supplemented with 2 µl FM4-64 (1 µg/ml) and 1% xylose to induce the expression of gpsB antisense RNA. Following a 20-minute incubation period in the microscope environmental chamber, the sample was imaged at 30 ºC, taking images every 15 minutes for a total time of 4 hours. Still imaging and time-lapse microscopy was completed on a DeltaVision Core microscope (Applied Precision/GE Healthcare) equipped with an environmental chamber, and photos were taken on a Photometrics CoolSnap HQ2 camera. For still imaging 17 planes were acquired every 200 nm while four planes were used for time-lapse microscopy. All data were deconvolved using SoftWorx software provided by the microscope manufacturer [154]. Structured illumination microscopy was completed using a DeltaVision OMX (Applied Precision/GE Healthcare) equipped with an OMX optical microscope (version 4) and a sCOMS camera.

**Immunoblot Analysis**

*S. aureus* cultures were grown overnight at 22 ºC in TSB supplemented with 15 µg/ml chloramphenicol or 5 µg/ml erythromycin where required for plasmid maintenance. On the following day, cultures were diluted 1:50 into fresh TSB containing appropriate antibiotics, and then grown to mid-logarithmic phase unless noted otherwise. 1 mM IPTG, 1% xylose, or 1.25 µM CdCl$_2$ was added directly to the culture medium (where required) to induce the expression of
gene(s) of interest or antisense RNA for three hours unless otherwise stated. Following the induction period 1 ml aliquots of samples were harvested and the bacterial cell pellet resulting from centrifugation was resuspended in 1 mL of a buffer containing 0.5 M sucrose, 20 mM MgCl₂, 10 mM KH₂PO₄, and 0.1 mg/ml lysozyme. Samples were then subjected to bead-beating with glass disruption beads (3 cycles, 30 seconds each) to complete the lysis process. Following bead-beating, glass disruption beads were removed from the samples via centrifugation. Samples were then separated by SDS-PAGE and transferred to nitrocellulose membrane that was subsequently probed with antisera against *S. aureus* GpsB or *B. subtilis* SigA, which was used as an internal loading control.

**Results**

**Expression of *S. aureus* gpsB in *B. subtilis* Results in Lethality and Cell Division Inhibition**

Previous works focusing on characterizing the function of GpsB in *B. subtilis* have indicated its role in guiding the localization of the class A penicillin binding protein, PBP1, to mid-cell positions during division events [122, 126, 127]. In order to determine if *S. aureus* GpsB (GpsB<sub>Sa</sub>) functions in a manner that is similar to the *B. subtilis* ortholog, we opted to express *gpsB<sub>Sa</sub>* and *gpsB<sub>Sa</sub>*-gfp in the laboratory *B. subtilis* strain, PY79, by placing them under the control of an inducible promoter. Strains were initially screened for growth defects associated with the expression of non-native *gpsB<sub>Sa</sub>* and *gpsB<sub>Sa</sub>*-gfp by streaking them on solid media in the absence and presence of the inducer. As controls, wild type PY79, and otherwise wild type *B. subtilis* strains harboring IPTG-inducible copies of either *B. subtilis* *gpsB* (*gpsB<sub>Bs</sub>*) or *gpsB<sub>Bs</sub>*-gfp were included in the analysis. In the absence of inducer all strains grew well and as expected (Figure 1, left panel). When strains were plated in the presence if 1 mM IPTG, however, strains expressing either *gpsB<sub>Sa</sub>* or *gpsB<sub>Sa</sub>*-gfp had significant growth defects indicating
that production of GpsB\textsubscript{Sa} was lethal in \textit{B. subtilis}. Wild type cells, and cells harboring an inducible copy of either \textit{gpsB}\textsubscript{Bs} or \textit{gpsB}\textsubscript{Bs}-\textit{gfp} grew similar to the corresponding strains in the absence of inducer (Figure 1 right panel).

![Figure 1](image)

**Figure 1.** Production of \textit{S. aureus} GpsB (GpsB\textsubscript{Sa}) in \textit{B. subtilis} results in a lethal phenotype. Plate phenotypes of wild type \textit{B. subtilis} (PY79) and otherwise wild type \textit{B. subtilis} strains harboring IPTG-inducible copies of either \textit{gpsB}\textsubscript{Sa} (GG7), \textit{gpsB}\textsubscript{Sa}-\textit{gfp} (GG8), \textit{B. subtilis} \textit{gpsB} (\textit{gpsB}\textsubscript{Bs}, GG18), or \textit{gpsB}\textsubscript{Bs}-\textit{gfp} (GG19) in the absence of inducer (left panel) or in the presence of 1 mM IPTG (right panel). Following inoculation, plates were incubated at 37 °C overnight, and observed for growth defects associated with \textit{gpsB} variant overexpression on the following day.

Next, to determine the source of the lethality described above, fluorescence microscopy was conducted to observe any morphological changes in \textit{B. subtilis} cells that were expressing \textit{gpsB}\textsubscript{Sa}. Inducer was added directly to mid-logarithmic phase \textit{B. subtilis} cultures in order to induce the expression of the \textit{gpsB}\textsubscript{Sa}. Uninduced cultures of the same strain were also included in this analysis to confirm that any phenotypic changes were specifically due to the production of GpsB\textsubscript{Sa} in \textit{B. subtilis}. Immunoblotting had revealed an approximate 3.2-fold overproduction of GpsB\textsubscript{Sa} when cells were grown in the presence of the IPTG inducer (Figure 2).

When grown in the absence of inducer, cells harboring an inducible copy of \textit{gpsB}\textsubscript{Sa} did not display any distinct phenotypes. The use of the red membrane stain, FM4-64, had allowed for the observation of regularly spaced division septa, and staining of the DNA with DAPI revealed properly segregated chromosomes (Figure 3A). Cells grown in the presence of inducer appeared significantly filamentous with irregular intervals between division septa, indicating that
cell division was inhibited (Figure 3B). These results had suggested that the lethality observed during the plate assay shown in Figure 1 when GpsB<sup>sa</sup> was produced in <i>B. subtilis</i> was potentially due to an inhibition of the cell division machinery.

![Figure 2. Immunoblot analysis of whole cell lysate harvested from <i>B. subtilis</i> wild type cells (PY79) or otherwise wild type cells harboring an inducible copy of gbps<sup>sa</sup> (GG7) grown in the absence or presence of inducer. Samples were separated using SDS-PAGE and subsequently transferred to nitrocellulose membrane that was probed with antibodies raised against GpsB<sup>sa</sup> or <i>B. subtilis</i> SigA (loading control). Note that antibodies against GpsB<sup>sa</sup> are unable to detect GpsB<sup>bs</sup>.

GpsB is known to interact with different cell division factors in addition to PBPs in a variety of Gram-positive bacteria such as <i>B. subtilis</i>, <i>L. monocytogenes</i>, and <i>S. pneumoniae</i> [126]. In order to gain further insight into the filamentation that we observed upon expression of gbps<sup>sa</sup> in <i>B. subtilis</i>, we engineered strains to express gbps<sup>sa</sup> in both a <i>ΔezrA</i> and <i>ΔponA</i> background, which encode known elements of the <i>B. subtilis</i> divisome that interact directly with the native version of GpsB. In addition, we also completed the analysis by expressing gbps<sup>sa</sup> in a <i>ΔprkC, ΔgpsB<sup>bs</sup></i>, and <i>ΔdivIVA</i> background. In the absence of inducer, all strains resembled the wild type control (Figure 3 A, C, E, G, I, K). Expression of gbps<sup>sa</sup> in all strain backgrounds resulted in severe filamentation despite the absence of the genes encoding various known GpsB interaction partners (Figure 3 D, F, H, J, L). It is important to note that absence of <i>divIVA</i> resulted in filamentation independent of gbps<sup>sa</sup> expression in the minus inducer control (Figure 3K).
Figure 3. Production of GpsBSa in B. subtilis results in severe filamentation. Cell morphology of B. subtilis strains harboring an inducible copy of gpsBSa in a wild type background (GG7) or in strain backgrounds harboring deletions of ezrA (GG35), ponA (CS26), prkC (CS24), gpbBSBs (CS40), or divIVA (CS94) grown in the absence (A, C, E, G, I, K) or presence (B, D, F, H, J, L) of inducer. Fluorescence of the membrane stain FM4-64 (red) and DAPI (blue) is shown. Scale bar is 1 µm.
Next, in order to determine if the filamentation caused by GpsB<sub>Sa</sub> production in <i>B. subtilis</i> was due to defects in Z-ring formation, we expressed <i>gpsB<sub>Sa</sub></i> in a <i>B. subtilis</i> strain background harboring a copy of <i>ftsZ-gfp</i> under the control of a constitutive promoter at an ectopic locus. In the absence of inducer, Z-rings were observed at regular intervals at mid-cell positions as expected (Figure 4A). In the presence of inducer, when <i>gpsB<sub>Sa</sub></i> was expressed, cells formed long filaments, as was previously observed (Figure 3B). However, FtsZ was diffused throughout the cytoplasm of the filamentous cells, indicating that filamentation associated with <i>gpsB<sub>Sa</sub></i> production in <i>B. subtilis</i> was specifically due to an inhibition of Z-ring assembly (Figure 4B).

**Figure 4.** GpsB<sub>Sa</sub> mediated filamentation in <i>B. subtilis</i> is due to a defect in Z-ring assembly. Cell morphology of a <i>B. subtilis</i> strain harboring an inducible copy <i>gpsB<sub>Sa</sub></i> and a copy of <i>ftsZ-gfp</i> under the control of a constitutive promoter at an ectopic locus (GG9). Cells were imaged following growth in the absence (A) or presence (B) of inducer. Fluorescence of the red membrane stain FM4-64 (red) and GFP (green) is shown. Scale bar is 1 µm.

**Altered GpsB<sub>Sa</sub> Levels in <i>S. aureus</i> Affect Cell Division and Cell Viability**

Next, as a method to further investigate the role of GpsB<sub>Sa</sub> in its native organism, we constructed a strain to overproduce GpsB<sub>Sa</sub> in <i>S. aureus</i> to determine if cell division would be inhibited, similar to what was observed in <i>B. subtilis</i>. Briefly, <i>gpsB<sub>Sa</sub></i> was cloned into a plasmid placing the gene of interest under the control of an inducible promoter. Cells were grown until mid-logarithmic phase and subsequently induced for 3 hours in order to overexpress <i>gpsB<sub>Sa</sub></i>. Following the induction period, cells were imaged along with wild type and empty vector controls in order to detect cell morphology changes associated with <i>gpsB<sub>Sa</sub></i> overexpression.
Expression of \(gpsB^{Bs}\) in \(S. aureus\) was also included in this analysis in order to determine if expression of the \(B. subtilis\) version in \(S. aureus\) would also result in phenotypic changes when compared to wild type and empty vector controls. Prior to running the microscopy, immunoblot analysis had revealed an approximate 5.4-fold overproduction of GpsB\(^{Sa}\) in the induced cultures (Figure 5).

\[
S. aureus
\]
\[
P_{\text{inducer}}^{-gpsB^{Sa}}
\]

\[
\begin{array}{ccc}
\text{WT} & - & + \text{ inducer} \\
\text{GpsB} & \text{Sample} & \text{Sample} \\
\text{SigA} & \text{Sample} & \text{Sample}
\end{array}
\]

**Figure 5.** Immunoblot analysis of whole cell lysate harvested from \(S. aureus\) wild type cells (SH1000) or otherwise wild type cells harboring a plasmid based inducible copy of \(gpsB^{Sa}\) (pPE45) grown in the absence or presence of inducer. Samples were separated using SDS-PAGE and subsequently transferred to nitrocellulose membrane that was probed with antibodies raised against GpsB\(^{Sa}\) or \(B. subtilis\) SigA (loading control).

Fluorescence microscopy and cell diameter quantification had revealed that 100% of wild type cells measured less than 1.2 \(\mu m\) where \(n=676\) (Figure 6A-B). As expected, the empty vector control also appeared similar to wild type as 100% of cells were less than 1.2 \(\mu m\) in diameter where \(n=100\) (Figure 6C-D). When grown without inducer, cells harboring an inducible copy of \(gpsB^{Sa}\) appeared similar in size to the wild type and empty vector controls as 93.6\% of cells were less than 1.2 \(\mu m\) in diameter where \(n=971\) (Figure 6E). In the presence of inducer, when \(gpsB^{Sa}\) was overexpressed, cells appeared significantly enlarged and 56.9\% of the cells were larger than 1.2 \(\mu m\) in diameter where \(n=770\), a hallmark of cell division inhibition in \(S. aureus\) (Figure 6F). Of note, expression of \(gpsB^{Bs}\) in \(S. aureus\) did not result in cell...
enlargement, indicating that the cell division inhibition phenotype was specific to the production of GpsB<sub>Sa</sub> (Figure 6G-H).

In addition to observing <i>S. aureus</i> cells overproducing GpsB<sub>Sa</sub> (from this point forward, GpsB) for cell morphology changes, we engineered a GpsB depletion strain to gain further insight into the essentiality of this protein. To do so, we placed <i>gpsB</i> antisense RNA under the control of an inducible promoter. Cells were grown to mid-logarithmic phase and then prepared for time-lapse fluorescence microscopy as described in the Materials and Methods section. An empty vector control was also used during this experiment in order to ensure that any changes in cell morphology, division, or viability were specifically due to a depletion of GpsB. Immunoblotting had revealed that production of antisense RNA against GpsB had resulted in an ~ 2.4-fold reduction in intracellular GpsB levels (Figure 7A).

![Figure 6](image)

**Figure 6. Overproduction of GpsB<sub>Sa</sub> in <i>S. aureus</i> results in cell enlargement.** Cell morphology wild type <i>S. aureus</i> cells (SH1000) or otherwise wild type cells harboring an empty vector control (SH1000 pCL15), an inducible plasmid-based copy of <i>gpsB<sub>Sa</sub></i> (SH1000 pPE45), or an inducible plasmid-based copy of <i>gpsB<sub>Bs</sub></i> (SH1000 pPE83) grown in the absence (A, C, E, G) or presence (B, D, F, H) of inducer. Fluorescence of the membrane stain FM4-64 (red) is shown. Scale bar is 1 µm.

Fluorescence microscopy had shown normal growth and division in cells harboring inducible antisense RNA against GpsB within the first 20 minutes of inducer exposure, similar to what was observed in the vector control. Images collected at later time points, however, revealed
morphological changes and defects observed only in cells where GpsB levels were depleted. These changes included clumping of the cell membrane, incomplete cytokinesis, aberrant division septa formation, and eventually cell lysis (Figure 7 B-C). The empty vector control continued to grow and divide normally throughout the course of the time-lapse experiment (Figure 7C). Collectively, these data indicate that S. aureus cells are sensitive to changes in GpsB levels, and that alterations result in morphological changes that are associated with cell division and viability. Furthermore, our results further confirm the previous conclusion that GpsB is an essential protein in S. aureus [145].

Figure 7. Depletion of GpsB in S. aureus is lethal. (A) Immunoblot analysis of whole cell lysate harvested from S. aureus cells containing an inducible copy of gpsB antisense RNA (left, SH1000 pGG59), or empty vector (right, SH1000 pEPSA5) grown in the presence of inducer. Samples were separated using SDS-PAGE and subsequently transferred to nitrocellulose membrane that was probed with antibodies raised against either S. aureus GpsB or B. subtilis SigA (loading control). (B-C) Time-lapse microscopy showing the cell morphology of S. aureus cells harboring either an inducible copy of gpsB antisense RNA (B, SH1000 pGG59), or empty vector (C, SH1000 pEPSA5). Times are indicated on top and the inducer was added at t=0. The white arrow indicates a cell that has initiated cell division at the time induction, and the white arrowhead indicates a cell that has not. Fluorescence of the membrane stain FM4-64 (red) is shown, scale bar is 1 µm.

GpsB Localization in S. aureus

We engineered a gpsB-gfp expressing S. aureus strain to monitor the localization of GpsB in actively dividing cells. Note that the level of GpsB-GFP production is lower here than in previous experiments where GpsB overproduction resulted in an inhibition of cell division. Cells harboring an IPTG inducible copy of gpsB-gfp were grown to mid-logarithmic phase and IPTG
was added, at a 0.5 mM final concentration, directly to the culture to induce the expression of \textit{gpsB-gfp}. Fluorescence microscopy revealed that GpsB-GFP localizes to mid-cell in cells undergoing an active round of cell division and to the cell periphery in those cells that are not. More specifically, GpsB-GFP that localized to mid-cell positions appeared to colocalize with the division septa (Figure 8).

![Membrane GpsB-GFP DNA +DNA](image)

**Figure 8.** GpsB-GFP localizes to mid-cell in actively dividing \textit{S. aureus} cells and to the cell periphery in those cells not actively dividing. Localization of GpsB in a \textit{S. aureus} strain harboring an inducible copy of \textit{gpsB-gfp} (SH1000 pPE46) grown in the presence of inducer for three hours. The white arrow shows mid-cell localization of GpsB, while the white arrowhead indicates GpsB localizing to the cell periphery in a non-dividing cell. Fluorescence of the membrane stain FM4-64 (red), GFP (green), and DAPI (blue) is shown. Scale bar is 1 µm.

Time-lapse fluorescence microscopy following a cluster of \textit{gpsB-gfp} expressing cells undergoing several rounds of cell division indicated a dynamic redistribution of GpsB-GFP during the division cycles. During septum formation GpsB-GFP localized to mid-cell positions as two foci, on either side of the division septa. Following septum closure, GpsB-GFP signal overlapped the division septa and stretched the diameter of the cells. GpsB-GFP was then observed along the periphery of the cells during the beginning of cytokinesis and following cell separation. In new daughter cells, GpsB-GFP then was redistributed back to mid-cell positions early on during septum formation (Figure 9).
Figure 9. Dynamic redistribution of GpsB-GFP during cell division in *S. aureus*. Time-lapse fluorescence microscopy showing the redistribution of GpsB-GFP within a cluster of *S. aureus* cells harboring an inducible copy of *gpsB-gfp* (SH1000 pPE46) at various stages of cell division. Time is indicated on the left, fluorescence of the membrane stain FM4-64 (red, left panel) and GFP (green, middle panel) is shown, and an illustration depicting GpsB-GFP localization at the various time points is shown on the right.
As a means to determine if mid-cell associated GpsB-GFP was localizing to the leading edge of the invaginating division septum, we utilized structured illumination microscopy (SIM) as previously described [42, 155]. During early cell division, GpsB-GFP was observed as two foci that colocalized with forming division septum (Figure 10i, left panels). Reconstruction of the deconvolved Z-stacks and rotation of the resulting image around the vertical-axis revealed that GpsB-GFP was forming a ring structure at mid-cell (Figure 10i, right panels). Furthermore, as septum maturation progressed, GpsB-GFP foci appeared to move closer together, closely following the leading edge of the division septum (Figure 10ii, left and right panels).

Figure 10. GpsB localizes to mid-cell as a ring structure and constricts with the division machinery. Structured illumination microscopy (SIM) monitoring GpsB-GFP localization in a S. aureus strain harboring an inducible copy of gpsB-gfp (SH1000 pPE46) at various stages of cell division (i: early septum formation, ii: septum maturation and constriction, iii: closure of the division septum, iv: completion of cell division). Left panels show cells at mid-plane, whereas the right panels show images produced from reconstructing deconvolved Z stacks and rotating the resulting image around the vertical axis. Fluorescence of membrane stain FM4-64 (red) and GFP (green) is shown.
Upon closure of the division septum, GpsB-GFP signal was observed as a single focus at the mid-point of the septum (Figure 10iii, left panels). Following the completion of cell division, GpsB-GFP began to redistribute to the periphery of the cell (Figure 10iv, left panels). Corresponding reconstructed and rotated images had revealed that the GpsB-GFP ring had constricted with the closure of the division septum (Figure 10iii-iv, right panels). Collectively, these data indicate that GpsB is highly dynamic during S. aureus cell division cycles. GpsB localizes to mid-cell as a ring structure early on during septum maturation, constricts with the invaginating division septum, and (upon septum closure and completion a division event) redistributes to the periphery of the cell prior to localizing again to mid-cell for the next division cycle.

**GpsB Depends on Functional FtsZ for Proper Localization to Mid-cell During Cell Division**

In most bacteria, the eukaryotic tubulin homolog FtsZ localizes to the division plane at the onset of cell division and forms a ring structure that serves as a scaffold in the recruitment of additional proteins to the divisome [8]. Thus cell division in various species of bacteria is often regulated through this central protein [156]. Being that overproduction of GpsB in S. aureus resulted in a block in cell division, and that GpsB-GFP localizes to division sites in actively dividing cells as a ring structure that constricts with the division septum, we were curious if GpsB localization was dependent upon functional FtsZ. To test this, we used the cell division inhibitor PC190723, which inhibits the GTPase activity of FtsZ [157, 158], in cells expressing $gpsB$-gfp as well as in empty vector control cells. In addition, we also used the inhibitor with a strain containing a copy of $zapA$-gfp, under the control of a cadmium-inducible promoter, in order to observe the effects of PC190723 on divisome formation. ZapA is another cell division protein that colocalizes early on in the division process as a ring structure with FtsZ and is often

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used a proxy to monitor Z-ring formation [74, 159, 160]. Cultures of the above listed strains were grown to mid-logarithmic phase in TSB supplemented with the appropriate antibiotics, and subsequently treated with 2 µg/ml of PC190723 for three hours. Cells were then harvested and prepped for microscopy as described in the Materials and Methods section. When grown in the absence of the drug, the empty vector control cells appeared of normal size and shape as 69.5% of cells had a diameter of 1.5 µm or less, n=200 (Figure 11A-A’). When treated with the drug for three hours, the diameter of the empty vector control cells was significantly increased with 92.5% of cells having a diameter of greater than 1.5 µm (n=200), indicating that cell division was being inhibited (Figure 11B-B’). As expected, PC190723 had a dramatic effect on divisome formation based on ZapA-GFP localization. In untreated cells, ZapA-GFP localized as concise rings at mid-cell positions in most cells indicating that the divisome was forming properly. When not observed at mid-cell, ZapA was found around the periphery of non-dividing cells (Figure 11C-C’). In contrast, in cells treated with PC190723, ZapA-GFP did not localize correctly in 96.5% of cells (n=200) and was observed as diffused signal (Figure 11D-D’). These data had indicated that cells treated with PC190723 experienced a cell division block due to defective divisome formation, likely due to defective FtsZ localization.

Next, in order to determine if GpsB requires functional FtsZ for proper localization to mid-cell in dividing S. aureus cells we examined the localization of GpsB-GFP in cells grown without and with PC190723 treatment. In untreated cells, GpsB-GFP localized to mid-cell in 55% of cells in a similar manner to what was observed previously (Figure 11E-E’ and Figure 8). GpsB-GFP was also observed to localize the cell periphery in 30% of cells and was observed as diffused signal in 15% of cells (Figure 11 E-E’, n=200). In contrast, in treated cells, GpsB-GFP was not localized properly in 100% of the cells observed (n=200). GpsB-GFP was
observed diffused in the cytoplasm of these cells (Figure 11F-F’). These data had indicated that GpsB is dependent on functional FtsZ for proper mid-cell localization in dividing S. aureus cells.

![Figure 11](image)

**Figure 11. Mid-cell localization of GpsB-GFP is dependent upon functional FtsZ.** (A-B’) Cell morphology of a S. aureus strain harboring a vector control (SH1000 pCL15) grown in the absence (A-A’) or presence (B-B’) of 2 µg/ml of the FtsZ inhibitor PC190723.  (C-F’)

Localization of either ZapA-GFP (FtsZ proxy, strain SH1000 pRB42) or GpsB-GFP (strain SH1000 pPE46) in cells grown in the absence (C-C’ and E-E’) or presence (D-D’ and F-F’) of 2 µg/ml PC190723. White arrows indicate normal ZapA rings. Fluorescence of GFP (green) is shown, scale bar is 1 µm.

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**FtsZ Localization is Sensitive to Altered Levels of GpsB**

We next wanted to test if FtsZ localization and divisome formation was influenced by GpsB in dividing *S. aureus* cells. To do so, we examined ZapA-GFP localization in cells either overproducing *gpsB* or in cells where intracellular GpsB levels were depleted. Overproduction of *gpsB* in *S. aureus* was observed to result in cell enlargement compared to vector and wild type controls (Figure 6E-F) indicating that cell division was being inhibited. We hypothesized that this phenotype was due to defective FtsZ localization and ultimately divisome formation. In a control stain expressing *zapA-gfp* under the control of an inducible promoter, ZapA-GFP was observed to localize to mid-cell in 53% of cells that were actively undergoing cell division (Figure 12A-A’, n=200). Similar localization of ZapA-GFP was observed in cells also containing a copy of *gpsB* under the control of an alternative inducible promoter when *gpsB* was not expressed in the absence of inducer (Figure 12 B-B’). Interestingly, when cells were grown in the presence of inducer allowing for overexpression of *gpsB*, ZapA-GFP did not localize correctly in 86% of cells (n=100) that also had an increased cell diameter (Figure 12 C-C’). This had indicated that GpsB-mediated cell enlargement in *S. aureus* was a result of impaired divisome formation.

In order to determine the effects on divisome formation in *S. aureus* cells where GpsB levels were depleted, we also monitored ZapA-GFP localization in a strain expressing *gpsB* antisense RNA under the control of an inducible promoter. In a vector control also containing an inducible copy of *zapA-gfp*, ZapA-GFP localized to mid-cell in 82.5% of cells (n=200) as expected (Figure 12D-D’). In contrast, in cells where GpsB levels were depleted, ZapA-GFP was observed as diffused signal in 59% of cells (n=200), indicating that reduced levels of GpsB had a significant impact of FtsZ localization and divisome formation (Figure 12E-E’). It is
interesting to note that the ZapA-GFP rings that did form in cells where GpsB levels were depleted appeared faint compared to those in the vector control (Figure 12D–D’ and Figure 12E–E’). Next, we quantified the fluorescence signal of the ZapA-GFP rings that did form in both the vector control and in cells where gpsB antisense RNA was expressed. We found a four-fold decrease in ZapA-GFP fluorescence signal in cells expressing gpsB antisense RNA when compared to the ZapA-GFP signal observed in rings in the vector control (2429 ± 1346 arbitrary units/cell, n=75; 614 ± 450 arbitrary units/cell, n=75) indicating that although ZapA rings were forming in these cells, there was an accumulation defect. Taken together with the results from the preceding section, these data indicate that FtsZ and GpsB influence each other’s localization in dividing S. aureus cells.

Figure 12. FtsZ fails to localize properly in dividing S. aureus cells with altered levels of GpsB. (A–C’) Localization of ZapA-GFP in wild type S. aureus cells (A–A’, SH1000 pRB42) and in a strain harboring an IPTG-inducible copy of gpsB (SH1000 pPE45 pRB42) grown in the absence (B–B’) or presence (C–C’) of 1mM IPTG. (D–E’) ZapA-GFP localization in a vector control strain (D–D’, SH1000 pEPSA5 pRB42) or S. aureus cells where GpsB levels were depleted through the expression of gpsB antisense RNA (E–E’, SH1000 pGG59 pRB42). White arrows indicate sites of cell division, whereas arrow heads indicate where GpsB-GFP is still localized to the subsequent division plane. Fluorescence of the membrane stain FM4-63 (red) and GFP (green) is shown. Scale bar is 1 µm.
**Discussion**

While most bacterial cell division studies have been completed in the rod-shaped model organisms *E. coli* and *B. subtilis*, recent work in non-model systems has shed light on various ways in which these organisms regulate the division process [128]. Here, we begin to characterize the role of a conserved cell division protein amongst the Firmicutes phylum, GpsB, in *S. aureus*. GpsB was initially characterized in *B. subtilis* where it was determined to be involved in cell wall synthesis and cell cycle regulation through mediating the proper localization of PBP1 [126]. Our results suggest that *S. aureus* GpsB has adopted additional roles in influencing divisome assembly in this organism. This conclusion is supported by recent biochemical analysis of *S. aureus* GpsB [136], as well as several key findings following our own cytological characterization of this protein.

Expression of *S. aureus* gpsB in *B. subtilis* resulted in severe filamentation that was due to defective Z-ring formation (Figure 3 and Figure 4). Furthermore, overproduction of *S. aureus* GpsB in its native species also resulted in a similar division block, which was not observed when *B. subtilis* gpsB was expressed in this organism (Figure 6). These data had indicated that the cell enlargement phenotype was specific to the *S. aureus* GpsB ortholog. Interestingly, this phenotype appeared similar to what was observed previously when FtsZ levels were depleted in *S. aureus* [161]. Comparing these phenotypes, it is possible to speculate how *S. aureus* GpsB may be influencing the activity of FtsZ *in vivo*. Interestingly, GpsB was recently determined play a role in the hydrolysis of GTP by FtsZ *in vitro*. In comparison to well-characterized *E. coli* FtsZ, *S. aureus* FtsZ was found to hydrolyze GTP relatively poorly below the concentration of 30 µM. The corresponding rate of GTP hydrolysis also remained low until FtsZ concentrations surpassed the 30 µM threshold [161-166]. Incubation of purified GpsB at a
concentration of 10 μM with 30 μM FtsZ was found to increase the GTP hydrolysis rate nearly three-fold [136]. Based on this, we hypothesize that the reason for GpsB-mediated cell enlargement in *S. aureus* may be due to increased FtsZ GTPase activity and ultimately an inhibition of functional Z-ring formation. Consistent with this hypothesis, we found that overproduction of GpsB in *S. aureus* negatively affects Z-ring formation, based on ZapA-GFP localization data. In a strain where *gpsB* was expressed, ZapA, which colocalizes with FtsZ early on in the division process [159, 160], failed to localize properly in a large population of the cells observed (*Figure 12C-C’*). In addition, depletion of GpsB, which resulted in cells with arrested cytokinesis (*Figure 7*), also resulted in a decrease in the magnitude of divisome formation (*Figure 12E-E’*).

GpsB-GFP localization data further supported the idea GpsB is interacting with the core division machinery in *S. aureus*. GpsB was observed to colocalize with the forming division septa as a ring, early on during cytokinesis, and co-constrict during the invagination process (*Figure 10*). Following the completion of cell division, GpsB-GFP was observed to redistribute to the periphery of the new daughter cells, and then to new sites of cell division preceding the next division event (*Figure 9*). Recent work had suggested a direct interaction between GpsB and purified FtsZ polymers [136]. Furthermore, transmission electron microscopy had revealed that GpsB promotes the formation of FtsZ polymer filaments when in combination with FtsZ polymers and GTP, which was similar to the FtsZ bundling activity that was previously observed in *E. coli* [75, 136, 167]. Lateral interactions were also previously observed when GpsB and FtsZ were in the presence of a GTP analog (GMPCPP), which is unable to be hydrolyzed and promotes the formation of filamentous FtsZ polymers. GpsB-mediated FtsZ organization was further confirmed through 90º angle light scattering [136]. We have observed that disruption of
Z-ring formation though the use of the FtsZ inhibitor PC190723 resulted in aberrant GpsB-GFP localization. Taken together with our observations regarding the effects of GpsB levels on divisome formation discussed above, these data suggest the ability for GpsB and FtsZ to influence each other’s ability to localize to the division plane. This observation further supports the idea of a direct interaction between GpsB and FtsZ polymers.

Based on results collected from our laboratory in combination with recent biochemical analysis of *S. aureus* GpsB indicating a direct interaction with the core division machinery [136], we propose a model in which GpsB promotes lateral interactions between FtsZ protofilaments. At a molecular level we show that, during cell division, FtsZ monomers undergo GTP-dependent polymerization into filaments that are subsequently bundled laterally by a direct interaction with GpsB at the division plane. We speculate that this interaction between GpsB and FtsZ increases FtsZ concentrations at the division plane, and thereby stimulates FtsZ GTPase activity allowing its disassembly (Figure 13A).

![Figure 13A](image)

**Figure 13. GpsB facilitates FtsZ remodeling during *S. aureus* cell division.** (A) Molecular model of GpsB activity in *S. aureus*. Green indicates FtsZ, either in monomeric or polymerized form as noted, and red indicates GpsB. (B) Cellular model of GpsB activity in *S. aureus*. Green indicates FtsZ and red indicates GpsB.
Supported by our observation that GpsB-GFP localization is influenced by functional FtsZ and divisome formation, we show FtsZ-mediated GpsB recruitment to the division plane early on. Lateral interactions between FtsZ protofilaments formed by GpsB promote FtsZ bunding, and localized increases in FtsZ concentration at mid-cell that stimulate the GTPase activity and ultimate disassembly of FtsZ. This in turn supports FtsZ treadmilling, which contributes to membrane invagination and cell wall synthesis at mid-cell during the division process in *S. aureus* (Figure 13B) [19, 20, 136, 161].
CHAPTER 5: FINAL DISCUSSION

Final Discussion

Chapter Overview

Although bacterial cell division studies have been ongoing in a variety of different species ranging from the rod-shaped model organisms, *E. coli* and *B. subtilis*, to non-model systems such as *S. aureus*, significant knowledge gaps still exist in how division is regulated both spatially and temporally [3, 128]. In both *B. subtilis* and *E. coli*, the well-studied Min and nucleoid occlusion systems have been found to be dispensable, as cell division continues unperturbed in their absence [51, 52]. This alludes to the existence of previously unidentified cell division regulators in these organisms. In *S. aureus*, recent work has focused on division site selection, cell wall synthesis, and cell shape maintenance [128, 129, 131, 168, 169]. Furthermore, *S. aureus* also lacks known cell division regulatory systems, such as the Min system, suggesting a drastically different mode of cell division regulation in this organism. In addition, how various bacterial species time the division process with external factors such as environmental stressors and nutrient availability, and with other essential cellular processes such as chromosome replication and segregation, is an active area of research [3, 170-172]. The essentiality of cell division has also allowed for recent studies to focus on this process as a novel therapeutic target as one means to combat the rise in antibiotic resistance [3, 140, 141].

The main goal of this dissertation was to further our understanding of how cell division is regulated in the Gram-positive organisms *B. subtilis* and *S. aureus*. These organisms differ significantly in cell shape and morphology, and likewise utilize different mechanism to govern
division site selection [131, 168, 173, 174]. Chapters 2 and 3 focused on the function of YpsA in 
*B. subtilis* and, to a lesser extent, *S. aureus*. We showed in chapter 2 that YpsA is a growth rate-
dependent cell division inhibitor in both organisms, and that YpsA offers oxidative stress 
protection in *B. subtilis*, although how this protection is achieved has yet to be elucidated. 
Furthermore, we began to broadly explore several conserved amino acid residues critical for the 
cell division inhibition function of YpsA, some of which make up defining characteristics of the 
YpsA subclade of the SLOG (SMF/DprA/SLOG) protein superfamily to which YpsA belongs 
[175]. These data, which have been published previously [137], constituted the first report on 
the role of YpsA in cell division regulation.

In chapter 3 we used a spontaneous suppressor screen in *B. subtilis* to isolate mutants that 
suppressed a lethal YpsA overproduction phenotype. Using this screen, we were able to isolate 
four unique intragenic point mutations that disrupted the cell division function of YpsA. In 
addition, using the crystal structure of YpsA, we were able to further analyze these residues 
gaining deeper insight into their functional and structural importance. Using the same suppressor 
screen, we were also able to isolate an extragenic suppressor mutation in a previously 
uncharacterized gene, *yfhS*. As cells lacking *yfhS* were also unable to elicit YpsA-mediated 
lethality, we speculated about the possible connections between YpsA and YfhS in this chapter. 
Through this approach we also discovered that YfhS may play a role in cell size regulation as 
cells lacking *yfhS* were smaller than wild type in both cell length and width.

In chapter 4 we examined the function of GpsB in *S. aureus*. YpsA and GpsB (formerly 
YpsB) appear to be in a syntenous relationship within the Firmicutes phylum of bacteria [137]. 
The role of GpsB in cell wall synthesis and PBP shuttling has been identified in multiple 
different organisms including *B. subtilis, Streptococcus pneumoniae*, and *Listeria*
Interestingly, data collected from our laboratory as well as from collaborators, which has been published previously [136], indicates that GpsB has adopted additional roles in *S. aureus*. These roles include regulating the dynamics of the central cell division protein, FtsZ, during the *S. aureus* cell cycle. In this chapter we explored the morphological changes associated with *gpsB* overexpression and depletion of intracellular GpsB levels. In addition, we used high resolution fluorescence microscopy, as well as structured illumination microscopy (SIM), to examine the dynamic localization patterns of GpsB-GFP during cell division in *S. aureus*. Finally, we showed that GpsB and FtsZ influence each other’s localization. In combination with biochemical analysis of GpsB [136], we proposed a model of the cell division function of GpsB in *S. aureus* at both a molecular and cellular level.

**Chapter 2**

Filamentation in *B. subtilis* and cell enlargement in *S. aureus* are hallmarks of cell division inhibition in these organisms, as cells continue to grow and synthesize peptidoglycan in the absence of cytokinetic events [168, 173, 176]. Thus, our initial observations that YpsA overproduction resulted in severe filamentation and cell enlargement in *B. subtilis* and *S. aureus*, respectively, had suggested a role for YpsA in cell division. This role for YpsA was further confirmed in *B. subtilis* through our observation that YpsA-mediated filamentation was the result of defective Z-ring formation in a strain constitutively expressing *ftsZ-gfp* from an ectopic locus. Transcriptomics data that suggests that *ypsA* is upregulated following hydrogen peroxide treatment [177, 178], and our observation that YpsA offers oxidative stress protection is intriguing in the context of its cell division function, as it may suggest a link between the oxidative stress response and the cue to divide. It is known that bacterial cells extensively regulate the division process in response to various environmental stimuli and stressors, as well
as in coordination with other essential cellular processes, such as DNA replication [3, 170-172].

It is interesting to note that DprA, a well-studied DNA repair protein involved in recombination via RecA, is grouped into the same protein super family as YpsA (SLOG) [179, 180]. The interconnectedness of the oxidative stress response and the DNA damage induced SOS response has been well-studied in E. coli, as radicals generated following oxidative stress strongly compromise genome integrity resulting in a SOS-mediated delay in cell division [181]. Given this information, and results from our study, we speculate the possibility that YpsA may be playing a role in regulating cell division in response to oxidative stress, however further work is required to confirm this connection. In addition, results taken from this chapter indicate that YpsA-induced filamentation is growth rate-dependent. Despite connections to oxidative stress and growth rate, an exact mechanism by which YpsA is eliciting its cell division inhibition function is unknown at this time.

Our analysis of several conserved amino acid residues included G53 and E55, both of which make up the conserved GxD/E motif important for substrate binding of YpsA proper subclade proteins based on structural and sequence analysis [175]. Through site-directed mutagenesis, we found that YpsA-GFP variants individually harboring G53A or E55Q point mutants did not result in filamentation when overproduced. Furthermore, we also found that YpsA-GFP foci formation was also disrupted. We speculate that foci formation is thus indicative of substrate binding and possibly multimerization, and thus disruption of this conserved motif results in the inability of YpsA to bind its substrate. This evidence suggested that foci formation is linked to the cell division inhibition function of YpsA. Our general characterization of other conserved amino acid residues further supports this link. YpsA-GFP variants individually harboring G42A, E44Q, W45A, or W87A mutations were also unable to
elicit filamentation upon overproduction. Interestingly, while W45A and W87A also resulted in impaired foci formation similar to G53A and E55Q, G42A and E44Q did not. We thus propose that foci formation is a prerequisite for YpsA-mediated cell division inhibition. Foci formation, however, does not necessarily imply that cell division is actively inhibited as we have observed cells containing multiple foci at wild type lengths. Further analysis of residues important for YpsA structure and/or function is included in the discussion section of chapter 3.

Based on the analysis above, the ability for YpsA to bind substrate appears to be important for YpsA to carry out its cell division inhibition function. In terms of what that substrate is, a connection between the YpsA proper subclade of the SLOG protein family and NAD or ADP-ribose has been previously made [175]. It is interesting to note that ADP-riboylation of FtsZ has been found to inactivate this central cell division protein, as was discussed in chapter 2 [182, 183]. However, we have not yet confirmed whether or not YpsA binds NAD or one of its derivatives, such as ADP-ribose. We could further tie in the growth rate dependency of YpsA-induced filamentation and the ability of YpsA to protect against hydrogen peroxide-induced oxidative stress to NAD through already established links [184, 185].

Based on the results from this initial work characterizing the function of YpsA discussed above and in chapter 2, the logical next steps in the progression of this study are to identify the substrate(s) of YpsA and to identify interaction partners of YpsA as a method to further elucidate the pathway in which it is functioning. The binding affinity of YpsA to NAD, or to other potential substrates, could be accomplished through assays such as isothermal titration calorimetry (ITC) [186-188]. This technique would require the purification of B. subtilis YpsA, which our lab has already successfully accomplished. Determination of whether or not YpsA
preferentially binds NAD, or one of its derivatives, would significantly contribute to a model where substrate binding is a prerequisite for apparent cell division inhibition function of YpsA.

Identification of putative interaction partners of YpsA could be accomplished through a FLAG immunoprecipitation using YpsA-FLAG as bait. In addition, YpsA-GFP-FLAG could also be used as bait to identify differences in interactions that may be due to the GFP tag. Identification of protein complexes within each of the samples would be completed via mass spectrometry. Furthermore, untagged YpsA could be used as a negative control, and as a means to subtract out any potential interactions that are non-specific. We have already conducted a pilot experiment [189] and are in the process of independently validating the interaction partners. Elucidation of interaction partners of YpsA would shed light on the pathway(s) in which YpsA is functioning, and thus further contribute to an overall model.

Chapter 3

The spontaneous suppressor screen described in chapter 3 of this dissertation allowed for the isolation of four unique intragenic suppressor mutations: G132E, P79L, E55D, and R111P. Each of these mutations disrupted the lethal phenotype previously observed upon YpsA-GFP overproduction when strains were plated on solid media in the presence of inducer. In addition, these mutations also resulted in cell lengths similar to the wild type control when grown in a liquid medium in the presence of inducer. These results indicate a link between YpsA-mediated cell division inhibition and the lethal phenotype observed when YpsA is overproduced, and thus when cell division inhibition is prevented no lethality is observed on solid medium. Notably, the G132E, P79L, and R111P mutations also disrupted YpsA-GFP foci formation. Keeping with the idea that foci formation is dependent on substrate binding and potentially indicative of multimerization as described above, this would suggest that these mutations inhibit the ability of
YpsA to bind substrate and thus YpsA cannot elicit its cell division inhibition function. Substrate binding, however, does not necessarily mean that cell division is actively being inhibited, as was discussed in chapter 2. Structural analysis using the crystal structure of YpsA allowed for us to provide meaning to intragenic suppressor mutations that we isolated during this study, which is thoroughly discussed in chapter 3. The residue E55 is highly conserved amongst the YpsA subclade of the SLOG protein super family and lines the predicted substrate binding pocket. We have previously mutated this residue in chapter 2, via site directed mutagenesis, to generate a E55Q mutation, and have observed a disruption in both foci formation and cell division inhibition upon overproduction of YpsA-GFP. Being that this residue lined the substrate binding pocket of YpsA, we speculated that it disrupted YpsA substrate binding, and thus inactivated YpsA in terms of its cell division inhibition function. As described above, the E55D mutation did not result in an impairment of foci formation but did prevent YpsA from inhibiting cell division upon overproduction. These data would suggest that YpsA harboring a E55D mutation is still able to bind substrate, at least at some capacity, however this mutation still inactivates YpsA. The inactivation of YpsA in this respect is potentially through changes in structure only. As it was discussed in chapter 3, the E55D mutation results in a shorter side chain and impairs hydrogen bonding to neighboring resides.

Our spontaneous suppressor screen also resulted in the isolation of an extragenic mutation in a previously uncharacterized gene, yfhS, that resulted in a premature stop codon. Our results indicated that cells lacking yfhS were unable to undergo YpsA-mediated filamentation and thus lethality associated with this phenotype was prevented. Transcriptomics data suggests that yfhS is upregulated during sporulation, and thus it was previously annotated as a sporulation gene [177, 178, 190]. Our finding that cells harboring a yfhS null mutation are
smaller in terms of length and width, when compared to wild type, suggests a role for YfhS during vegetative growth. Furthermore, we have noted that cells lacking yfhS grow slower than wild type, as described in chapter 3. Based on our findings from chapter 2, it is possible that cells harboring yfhS null mutations are unable to undergo YpsA-mediated filamentation and associated lethality due to a decreased growth rate. Complementation of yfhS from an ectopic locus seems to suggest that this may be the case as growth rate is restored. As was stated in chapter 3, however, we have not yet ruled out a direct connection between YfhS and YpsA, and thus this is the primary focus of our follow-up study. The potential role of YhfS in cell size regulation makes the possible connection to YpsA (either direct or indirect) favorable as the coordination between cell size and cell division has previously been established [191].

**Chapter 4**

In combination with biochemical analysis by collaborators [136], results obtained from our laboratory suggest that GpsB in *S. aureus* plays a role in cell division by regulating the dynamic behavior of the cell division protein FtsZ. Our finding that overproduction of *S. aureus* GpsB in both *B. subtilis* and *S. aureus* results in cell division inhibition, while the *B. subtilis* ortholog is unable to elicit this activity in either organism, strongly suggests that this phenotype is specific to the *S. aureus* version. Furthermore, we have shown that overproduction of *S. aureus* GpsB in *B. subtilis* is lethal when the overproduction strain was plated on solid medium in the presence of inducer, although spontaneous colonies were still able to develop that presumably contained suppressor mutations similar to what is described in chapter 3. The utilization of a suppressor screen to identify intragenic suppressor mutations or extragenic suppressor mutations is an area of active investigation within our laboratory.
The proposed function of GpsB in *B. subtilis, L. monocytogenes*, and *S. pneumoniae* involves a direct interaction with PBPs involved in cell wall synthesis [126]. While our study in *S. aureus* constitutes the first suggestion of a direct interaction between GpsB and FtsZ, it is still possible for GpsB to retain its ability to function in the cell wall synthesis pathway. We are thus testing the possibility of such interactions through bacterial two-hybrid and *in vitro* assays such as surface plasmon resonance with purified proteins [143]. We can follow up this study with an unbiased approach using an immunoprecipitation assay with GpsB-FLAG as bait and identify protein complexes via mass spectrometry as was described for YpsA in chapter 2. Interactions between GpsB and proteins identified via mass spectrometry will be validated independently. Confirmation of additional GpsB interactions would significantly contribute to our overall model, potentially linking the function of GpsB in regulating FtsZ dynamics to other cellular processes.

Previous works in *B. subtilis* and *S. pneumoniae* indicate an inherent link between GpsB activity and serine threonine (Ser/Thr) protein kinases [42]. In *B. subtilis*, the activity of the Ser/Thr kinase, PrkC, is stimulated by GpsB at the division septum. This in turn allows for GpsB phosphorylation. Phosphorylated GpsB was determined to be unable to further stimulate PrkC, thus establishing a negative feedback loop regulating its activity [192]. In *S. pneumoniae*, an interaction between GpsB and StkP mediates the phosphorylation of multiple cell division proteins, including DivIVA. Furthermore, GpsB in complex with StkP may also aid in governing the switch between peripheral and septal peptidoglycan synthesis [144]. Evidence in *S. aureus* also suggests the possibility of an interaction between GpsB and the Ser/Thr kinase, Stk1 (PknB). *S. aureus* GpsB is known to be phosphorylated at multiple sites [126]. In addition, a role for Stk1 in cell division has already been implicated. Previous work has shown division
site localization of Stk1 as well as the phosphorylation of FtsZ by this Ser/Thr kinase [193].

Based on what is known in *B. subtilis* and *S. pneumoniae*, we speculate that GpsB phosphorylation in *S. aureus* dictates its activity with respect to its cell division related functions. In order to test this, we will determine whether this kinase is able to phosphorylate GpsB *in vitro* using purified GpsB and Stk1. In parallel, we will analyze the significance of the predicted phosphorylation sites in terms of GpsB function [194] via site directed mutagenesis and constructing phospho-mimetic and phospho-ablative mutations.
REFERENCES


Deciphering the Role of a SLOG Superfamily Protein YpsA in Gram-Positive Bacteria

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Bacteria adapt to different environments by regulating cell division and several conditions that modulate cell division have been documented. Understanding how bacteria transduce environmental signals to control cell division is critical in understanding the global network of cell division regulation. In this article we describe a role for Bacillus subtilis YpsA, an uncharacterized protein of the SLOG superfamily of nucleotide and ligand-binding proteins, in cell division. We observed that YpsA provides protection against oxidative stress as cells lacking ypsA show increased susceptibility to hydrogen peroxide treatment. We found that the increased expression of ypsA leads to filamentation and disruption of the assembly of FtsZ, the tubulin-like essential protein that marks the sites of cell division in B. subtilis. We also showed that YpsA-mediated filamentation is linked to the growth rate. Using site-directed mutagenesis, we targeted several conserved residues and generated YpsA variants that are no longer able to inhibit cell division. Finally, we show that the role of YpsA is possibly conserved in Firmicutes, as overproduction of YpsA in Staphylococcus aureus also impairs cell division.

Keywords: FtsZ, GpsB, Bacillus subtilis, oxidative stress, cell division, NAD, SLOG

IMPORTANCE

Although key players of cell division in bacteria have been largely characterized, the factors that regulate these division proteins are still being discovered and evidence for the presence of yet-to-be-discovered factors has been accumulating. How bacteria sense the availability of nutrients and how that information is used to regulate cell division, positively or negatively, is less well-understood even though some examples exist in the literature. We discovered that a protein of hitherto unknown function belonging to the SLOG superfamily of nucleotide/ligand-binding proteins, YpsA, influences cell division in Bacillus subtilis, directly or indirectly, by integrating nutrient availability and growth rate. We also showed that YpsA is important for the oxidative stress response in B. subtilis. Furthermore, we provide evidence that the possible cell division inhibition function of YpsA is also conserved in another Firmicute, Staphylococcus aureus. This first report on the role of YpsA brings us a step closer in understanding the complete tool set that bacteria have at their disposal to precisely regulate cell division to adapt to varying environmental conditions.
INTRODUCTION
Cell division in bacteria is a well-orchestrated event that is achieved by the concerted action of approximately a dozen different key division proteins (Haenicke and Margolin, 2016). Amongst them is a protein central to cell division, a tubulin-like protein FtsZ, which marks the site of cytokinesis in most bacteria (Busiek and Margolin, 2015; Du and Lutkenhaus, 2017). In addition to standard spatial regulators of septum positioning (Eswara and Ramamurthi, 2017), factors that sense nutrient availability (Wang and Levin, 2009; Monahan et al., 2014a), DNA damage (Djakovic et al., 2008; Mo and Burkholder, 2010; Modell et al., 2014), and alternate external environments (Justice et al., 2008; Khandige et al., 2016), have been shown to influence cell division. The observation that cell division in model organisms Escherichia coli and Bacillus subtilis that lack well-studied Min and nucleoid occlusion regulatory systems undergo cell division largely unperturbed (Monahan et al., 2014b), and other reports that suggested Min system does not play a role in division site selection in B. subtilis (Gregory et al., 2008; Eswaramoorthy et al., 2011); prompted us to investigate the presence of other factors involved in cell division regulation. Here we describe the possible role of YpsA, a protein conserved in several members of the Firmicutes phylum, in cell division.

The genes ypsA and gpsB (formally ypsB) are in a syntenous relationship in many Firmicute genomes (Figure 1A). GpsB is a cell division protein that regulates peptidoglycan synthesis in B. subtilis (Claessen et al., 2008; Tavares et al., 2008), Streptococcus pneumoniae (Fleury et al., 2014; Rued et al., 2017), and Listeria monocytogenes (Rusmondo et al., 2016). More recently, our group showed that Staphylococcus aureus GpsB affects the polymerization kinetics of FtsZ directly (Eswara et al., 2018). As genes in a syntenous arrangement across multiple genomes, often referred to as conserved gene neighborhoods, are commonly indicative of functional relationships (Aravind, 2009; Huyten et al., 2000), we were curious to study the function of YpsA in B. subtilis. Prior to our investigation, the crystal structure of B. subtilis YpsA was solved by a structural genomics group (PDB ID: 2NX2). Based on the unique structure and sequence features (Figure 1B), YpsA was classified as the founding member of the “YpsA proper” clade in the S undermine/DprA/LOG (SLOG) protein superfamily (Burroughs et al., 2015). The SLOG superfamily contains a specific form of the Rossmann fold, and is involved in a range of nucleotide-related functions. These include the binding of low-molecule weight biomolecules, nucleic acids, free nucleotides, and the catalyzing of nucleotide-processing reactions (Fischer et al., 2006; Mortier-Barrière et al., 2007; Samanovic et al., 2015). Recently, several members of the SLOG superfamily were further identified as key components in a newly-defined class of biological conflict systems centered on the production of nucleotide signals. In these systems, SLOG proteins are predicted to function either as sensors binding nucleotide signals or as nucleotide-processing enzymes generating nucleotide derivatives which function as signals (Burroughs et al., 2015). Despite these new reports, the precise function of YpsA and its namesake family have yet to be experimentally investigated.

Here we report that (i) YpsA provides protection against oxidative stress; (ii) overexpression of ypsA causes mislocalization of FtsZ-GFP that results in cell filamentation, which is dependent on the growth rate; (iii) YpsA-GFP forms dynamic foci that are possibly mediated by nucleotide binding; and finally (iv) overexpression of ypsA in S. aureus results in cell enlargement typical of cell division inhibition in cocci (Pinho and Errington, 2003)—suggesting a conserved function for YpsA across Firmicutes with very different cell-morphologies. In sum, these results constitute the first report on YpsA and its role in oxidative stress response and possibly in cell division regulation.

RESULTS
YpsA Provides Oxidative Stress Protection
As a first step to study the significance of YpsA, we experimentally investigated the phenotype of a ypsA null strain in several stress-inducing conditions through a standard disc-diffusion assay with compounds that induce: DNA damage (0.1 mM mitomycin C-treatment), membrane stress (1% SDS or 5 mg/ml daptomycin), general stress (a range of 10–50% ethanol), cell wall stress (commercially available discs with Penicillin 10 units and vancomycin 30 μg), oxidative stress (0.1 M, 0.5 M, or 1 M H2O2); as well as heat stress (growth of cells at 48°C). We noticed that the ypsA null strain exhibited a larger zone of inhibition in comparison to the wild type (WT) control, reproducibly, only when incubated with discs containing 0.5 M or 1 M H2O2 (WT: 1.8 ± 0.45 mm; ΔypsA: 7.6 ± 0.54 mm; Figure 2A), suggesting that YpsA provides protection against oxidative stress. It is noteworthy that ypsA transcript level is elevated upon hydrogen peroxide treatment ([Nicolas et al., 2012; Zhu and Stülke, 2018]; Figure S1). To further evaluate this phenotype, we monitored the cells grown in liquid culture in the absence or presence of 1 mM H2O2 using fluorescence microscopy (Figure 2B). Untreated cells lacking ypsA appear morphologically similar to WT (Figure 2B; see WT and ΔypsA left panels) and grew similar to WT in conditions tested (Figure 3A). Although WT cells were tolerant to H2O2 treatment, ΔypsA cells displayed obvious signs of a sick phenotype with cells that are en route to lysis when incubated with discs containing 0.5 M or 1 M H2O2 (WT: 23% sick cells, n = 100; ΔypsA: 79% sick cells, n = 100). To test if this phenotype is specifically due to absence of YpsA, we introduced an inducible copy of ypsA-gfp into WT in conditions tested (Figure 2B), and other reports that suggested Min system does not play a role in division site selection in B. subtilis (Gregory et al., 2008; Eswaramoorthy et al., 2011); prompted us to investigate the presence of other factors involved in cell division regulation. Here we describe the possible role of YpsA, a protein conserved in several members of the Firmicutes phylum, in cell division.
Figure 1 | (A) Left: Cartoon representation of ypsA gene neighborhood in Firmicutes, not to scale. The genes that encode protein products containing a domain of unknown function DUF1798 are named as such. Right: Phylogenetic tree of the YpsA family, key branches with >70% bootstrap support are denoted with yellow circles. Reproducible clades within the family are color-coded according to their phyletic distribution and labeled with names and representative conserved domain architectures and gene neighborhoods. For these genome context depictions, colored polygons represent discrete protein domains within a protein, while boxed arrows represent individual genes within a neighborhood. Each context is labeled with NCBI accession and organism name, separated by an underscore. For gene

(Continued)
Increased Production of YpsA Inhibits Cell Division

Next, we examined the ypsA overexpression phenotype. For this purpose, we constructed an otherwise WT-strain to ectopically express either ypsA or ypsA-gfp upon addition of inducer. Quantification of GFP fluorescence revealed that there was 3-fold overproduction of YpsA-GFP in the presence of inducer (2,415 ± 1,296 arbitrary units; n = 50) when compared to YpsA-GFP produced under the control of its native promoter (732 ± 692 arbitrary units; n = 50; RB221). We then monitored the cell morphology of cells overproducing YpsA or YpsA-GFP. To our surprise, as shown in Figure 3, when compared to the cell lengths of inducible strains grown in the absence of inducer [YpsA: 2.92 ± 0.81 μm (Figure 3A); YpsA-GFP: 3.89 ± 0.98 μm (Figure 3C); n = 100], cells overproducing YpsA or YpsA-GFP appeared elongated [YpsA: 8.92 ± 4.89 μm (Figure 3B); YpsA-GFP: 9.57 ± 4.99 μm (Figure 3D); n = 100] implying that cell division is inhibited by YpsA (Figure 3I). This result also indicated that the fluorescent protein tagged fusion of YpsA is functional reaffirming the conclusion derived in the previous section. Tracking fluorescence of YpsA-GFP in our overexpression strain showed that YpsA assembles into discrete foci (Figure 3D). To study whether YpsA is capable of self-interaction, based on the ability to form a focus, we conducted a FLAG tag-based...
immunoprecipitation assay in a strain producing both YpsA-GFP and YpsA-FLAG. Upon using YpsA-FLAG as a bait we were able to pulldown YpsA-GFP species indicating that YpsA is capable of forming a homocomplex (Figure S2). An unrelated protein, housekeeping sigma factor SigA, served as our negative control which was not present in the eluate fraction. To further analyze the YpsA-GFP foci in our overexpression strain, we conducted time-lapse microscopy at 2-min intervals for 6 min which revealed that YpsA foci are dynamic within a 1-μm range (Figures 3J–M; see arrows; also see the video taken at 1 min intervals in the Supplementary Information). Quantification of the YpsA foci revealed that on average there are 2, 4, or 9 foci per cell in cells that are smaller than 5 μm, between 5 and 10 μm, or larger than 10 μm, respectively (n = 100). Also, upon tracking 100 different foci, we observed short-range (<1 μm) mobility in 71% of the cells (n = 100). Since YpsA-GFP retains fluorescence as a focus and that the foci are mobile, and focus disruption occurs in some strains that express mutated versions of ypsA (Figure 6A), we conclude that the foci are not artifacts of non-functional misfolded aggregates. We also observed that foci formation occurs when YpsA-GFP production is driven from its native promoter and upon H2O2 treatment in 9% and 12% of the cells, respectively (Figure 2C).

Since genes coding for YpsA and the cell division protein GpsB are in a syntenous relationship, we aimed to test whether the YpsA overproduction-mediated filamentation is dependent on GpsB. As shown in Figures 3E–H, cells lacking gpsB also formed filaments upon overexpression of ypsA or ypsA-gfp, suggesting that YpsA-mediated cell division inhibition is independent of GpsB.

YpsA Overproduction Disrupts FtsZ Assembly

Typically, filamentation is a result of impaired FtsZ ring assembly. To test whether FtsZ ring assembly is affected by YpsA overproduction, we engineered strains that constitutively produces FtsZ-GFP (Gregory et al., 2008; Eswaramoorthy et al., 2011), to also produce either ypsA or ypsA-mCherry under the control of an inducible promoter. In FtsZ-GFP producing otherwise WT cells, the cell length appeared normal and FtsZ assembled into FtsZ rings at mid-cell in 90% of the cells (Figures 4A,B; top panels; n = 100). In the strains capable of producing both FtsZ-GFP and YpsA or YpsA-mCherry, when cells were grown in the absence of inducer, FtsZ-GFP localization appeared similar to the control strain (Figures 4A,B; middle panels). In striking contrast, when cells were grown in the presence of inducer FtsZ-GFP assembled into rings in only 8% or 3% of cells that express ypsA or ypsA-mCherry, respectively (Figures 4A,B; bottom panels; n = 100).
FIGURE 4 | YpsA inhibits FtsZ ring assembly. (A) Fluorescence micrographs of cells that either constitutively produce FtsZ-GFP in otherwise wild type strain (PE92; top panel) and cells that constitutively produce FtsZ-GFP and additionally harbor a copy of inducible ypsA (RB15) grown in the absence (middle panel) or presence of inducer IPTG are shown. Fluorescence of FM4-64 membrane dye (red) and GFP (green) are shown. (B) Cellular morphologies of cells that constitutively produce FtsZ-GFP (PE92) and cells that additionally contain a copy of inducible ypsA-mCherry (RB97) grown in the absence (middle panel) or presence of inducer are shown. DIC (gray) and fluorescence of GFP (green) and mCherry (red) are shown. (C) Cells of GG82 (inducible ypsA) and RB15 (inducible ypsA + constitutively expressed ftsZ-gfp) were imaged after grown in the presence of inducer for 1 h (top panels) or 3 h after the removal of inducer (bottom panels). Scale bars: 1 µm.

Resumption of Cell Division After Removal of Inducer

Next, we wished to test whether filamentous cells resume normal division following the removal of inducer. For this purpose, we grew the cells of the inducible ypsA strain with or without the FtsZ-GFP reporter until the mid-logarithmic phase (OD_{600} = 0.5). We then added the inducer to induce the production of YpsA. Monitoring the cell morphology of these cells 1 h post addition of the inducer, revealed filamentation in cells expressing ypsA (cell length: 9.17 ± 4.76 µm; Figure 4C top left panel; n =}
YpsA inhibits division in bacteria

**FIGURE 5** | Growth rate-dependent inhibition of cell division. (A) Fluorescence micrographs of cells of WT, inducible ypsA (GG82) or ypsA-gfp (GG83) were grown in CH medium in the absence of any supplements (top panels), with 1% glucose supplementation (middle panels), or with 1% sucrose supplementation (bottom panels). (B) WT, GG82, or GG83 strains described above were grown in LB medium at 37°C (top panels) or 22°C (bottom panels). Fluorescence of membrane dye (FM4-64; red), GFP (green) are shown. Scale bar: 1 µm.

In cells that also produce the FtsZ-GFP reporter, in addition to filamentation (cell length: 8.20 ± 5.17 µm; **Figure 4C** top right panel), we also observed disruption of the FtsZ-ring assembly (FtsZ rings: 11% in cells <5 µm and 4% in cells ≥5 µm). We then pelleted the cell culture and washed off the inducer with a fresh medium thrice. Following the growth of the cells in fresh medium, 3 h after the removal of the inducer, we observed that the cell lengths of ypsA expressing cells with or without the FtsZ-GFP reporter were 4.30 ± 2.14 µm and 5.66 ± 4.40 µm, respectively (**Figure 4C** bottom panels), indicating restoration to normal growth. Quantification of FtsZ rings revealed resumption of FtsZ ring assembly subsequent to the removal of inducer (FtsZ rings: 57% in cells <5 µm and 7.5% in cells ≥5 µm; **Figure 4C** bottom right panel). These observations suggest that normal division resumes in filamentous cells after the removal of the inducer.

**Sporulation Frequency Is Unaffected in ypsA Strains**

Since cotD, which codes for a spore coat protein, is immediately upstream of ypsA (**Figure 1A**), we were curious to see if ypsA has any role in sporulation. To address this, we performed a sporulation assay using Casein Hydrolysate (CH)-based growth medium and Sterlini-Mandelstam sporulation medium in triplicates (Eswaramoorthy et al., 2009). The average sporulation frequency of ΔypsA strain was 176% relative to WT (100%), which is a modest 2-fold increase in frequency, suggesting YpsA has no appreciable role in sporulation. To study whether YpsA overproduction-mediated filamentation impairs sporulation, we conducted a similar sporulation assay and found that cells overexpressing ypsA (98%) or ypsA-gfp (127%) also displayed a sporulation frequency similar to WT.

**YpsA Overproduction-Induced Filamentation Is Dependent on Growth Rate**

To fully comprehend how filamentous cells achieve WT-like sporulation efficiency, we observed the cell morphology of ypsA overexpressing cells grown in the presence of inducer in CH medium using fluorescence microscopy. Surprisingly, the cell lengths of ypsA or ypsA-gfp overexpressing cells appeared similar to WT when grown with or without the inducer (YpsA: 2.73 ± 0.69 µm vs. 3.17 ± 0.80 µm; YpsA-GFP: 3.02 ± 0.68 µm vs.
of inducer in ypsA-gfp expressing cells, although cells were not filamentous. YpsA-GFP foci formation was still observed (Figure 5A top right panel).

We hypothesized that the lack of nutrients in CH compared to LB might be the reason for the lack of filamentation. To test our hypothesis, we externally supplemented either 1% glucose or 1% sucrose to the CH medium. Intriguingly, mid-log phase cells (OD$_{600}$=0.5) grown in CH in the presence of glucose and inducer to overproduce YpsA or YpsA-GFP lead to filamentation (YpsA: 3.14 ± 0.65 µm vs. 7.93 ± 2.39 µm; YpsA-GFP: 3.02 ± 0.84 µm vs. 10.31 ± 5.26 µm; n = 100; Figure 5A middle panels). Similar results were observed when the CH medium was supplemented with sucrose (YpsA: 2.94 ± 0.64 µm vs. 9.53 ± 2.69 µm; YpsA-GFP: 2.87 ± 0.78 µm vs. 9.71 ± 3.51 µm; n = 100; Figure 5A bottom panels), suggesting that filamentation is dependent on nutrient availability.

To evaluate whether the filamentation is linked to nutrient availability or perhaps the growth rate, we monitored the growth rate by measuring the absorbance at 600 nm (OD$_{600}$) over the course of 5 h. We observed that supplementation of either glucose or sucrose increased the growth rate when compared to strains grown in CH medium without any supplementation (Figure S3B). As an independent analysis to study this phenomenon of growth rate-dependent cell division inhibition, we grew cells in LB medium at either 22 or 37°C. As expected, cultures grown at 22°C displayed a slower growth rate compared to cultures grown at 37°C (Figure S3C). Next, we imaged the mid-log phase (OD$_{600}$ = 0.5) cells of cultures grown at 22 or 37°C. While the cells grown in LB at 37°C displayed severe filamentation, as discussed in previous sections, the cells grown at 22°C displayed normal cell growth (Figure 5B bottom panels). We further verified the stable production of YpsA in slow growing cells by performing western blotting of cells harvested at mid-log phase and stationary phase (Figure S3D). Based on these results, it appears that YpsA is a growth rate-dependent cell division inhibitor.

A known factor that inhibits cell division depending on the presence of glucose is UgtP, which is a UDP-glucose diacylglycerol glucosyltransferase (Weart et al., 2007). Therefore, we tested if the YpsA-mediated filamentation is dependent on UgtP using a ugpP null strain. As shown in Figure S4, cells lacking ugpP also undergo filamentation upon increased production of YpsA suggesting that cell division inhibition by YpsA is independent of UgpP.

Identification of Amino Acid Residues Important for YpsA Function

Aided by the crystal structure and computational analysis of the YpsA family of SLOG domains, we identified several conserved residues that are predicted to be important for maintaining the function of YpsA (Figure 1B; see arrows). We performed site-directed mutagenesis of two of the key residues that were speculated to be involved in substrate binding and generated GFP-tagged ypsA variants G53A and E55Q. We also generated other mutants to more generally explore YpsA
function including G42A, E44Q, W45A, W57A, and W87A. We ensured that all mutants were stably produced through immunoblotting (Figure 6B). Fluorescence microscopy-based examination revealed that all YpsA variants except W57A were unable to trigger filamentation upon overexpression (Figure 6A), suggesting that YpsA function is compromised in all of these mutants. We also noticed that G53A, E55Q, W45A, and W87A mutants displayed impaired ability to form foci. This is consistent with the observation that the first two of these mutations disrupt the conserved, predicted nucleotide-binding site of the YpsA family (Burroughs et al., 2015), and the latter two likely disrupt a key strand and helix of the Rossmannoid fold.

**Overproduction of YpsA Inhibits Cell Division in S. aureus**

To investigate if the role of YpsA is conserved in other Firmicutes, we chose to study the function of YpsA in *S. aureus*. Cells lacking intact *ypsA* in *S. aureus* (Fey et al., 2013), are viable and their cell morphology appear similar to WT control suggesting that, at least in the conditions tested here, *ypsA* is not an essential gene (Figure 7A). Next, we placed *S. aureus* *ypsA* (*ypsA*Δ) under the control of a xylose-inducible promoter in a *S. aureus* plasmid vector. Cells capable of producing *ypsA*ΔA or containing an empty vector control were then grown to mid-log phase (OD₆₀₀ = 0.5) at 37°C at which point the inducer was added to produce YpsAΔA and culture samples for microscopy were harvested at the 1 h timepoint. As shown in Figure 7B, 1 h after the addition of inducer, the cell diameter of the WT control (0.88 ± 0.16 µm; n = 100; Figure 7A) and the vector control strain grown in the absence of inducer (0.88 ± 0.16 µm) and presence of inducer (0.89 ± 0.18 µm), resembled the inducible *ypsA*ΔA strain grown in the absence of inducer (0.89 ± 0.17 µm; Figure 7C). Interestingly, cells overexpressing *ypsA*ΔA were unable to undergo septation and displayed clear cell enlargement (1.72 ± 0.37 µm; n = 100; Figure 7C), a telltale sign of cell division inhibition in this organism. Therefore, the possible function of YpsA in inhibiting cell division is conserved in *S. aureus*, and perhaps in other Firmicutes which code for it despite the differences in their cell morphologies.

Next, we wondered whether the enlarged cells could revert back to normal growth upon removal of the inducer, similar to what was observed in *B. subtilis* (Figure 4). For this purpose, we centrifuged the cell culture and washed the inducer off the cell pellet with fresh medium three times and resuspended in fresh medium. Three hours after the removal of inducer, septation appears to have resumed, albeit with errors in septum positioning (see arrow in Figure 7C). The average diameter of the cells returned closer to WT-like (1.08 ± 0.35 µm; n = 100), indicating that enlarged cells overproducing YpsA could revert back to normal growth provided that the YpsA level is reduced. To test if cell division inhibition in *S. aureus* is also dependent on growth rate, we grew the inducible *ypsA*ΔA strain in the presence of inducer at 22°C. Similar to what we observed in *B. subtilis* (Figure 5B), when *S. aureus* cells capable of overproducing YpsAΔA were grown in the presence of inducer at 22°C, we did not observe cell enlargement (0.92 ± 0.17 µm; Figure 7C).
DISCUSSION

Bacterial cell division is a highly regulated process and many division factors have already been characterized especially in the model organisms *E. coli* and *B. subtilis*. Yet, cell division is only mildly affected even in the absence of a combination of known division regulators in these organisms (Monahan et al., 2014b), thus predicting the presence of other proteins that could affect the cell division process. Here, we discuss the role of YpsA, a protein of hitherto unknown function conserved in diverse Firmicutes. We show that YpsA offers protection against oxidative stress. However, the precise mechanism of how this is achieved remains to be elucidated. Next, we show that YpsA overproduction leads to impaired FtsZ ring assembly and ultimately cell division inhibition—although this could potentially be an indirect effect.

It has been reported that the *cotD-ypsA* transcriptional unit is repressed by the regulator essential for entry into sporulation, Spo0A (Fujita and Losick, 2003), which binds to a region upstream of *cotD* (Molle et al., 2003). It has been shown that *cotD* is also repressed by a late stage sporulation-specific transcriptional regulator, SpoIID (Halberg and Kroos, 1994). Both *cotD* and *ypsA* transcripts are at similar levels in various growth conditions except in those that promote sporulation [Figure S1; (Nicolas et al., 2012; Zhu and Stülke, 2019)]. The function of CotD during normal growth, if any, needs to be evaluated. It has been reported that *cotD* level increases in a concentration-dependent manner in response to antibiotic treatment (Lin et al., 2005). In this report we show that cells lacking *ypsA* or overexpressing *ypsA* show no obvious sporulation defect and that YpsA-mediated cell division inhibition is dependent on the growth rate. Other reports exist that describe the connection between nutrient availability, growth rate, and cell division (Yadav and Levin, 2015; Westfall and Levin, 2017). At this time, we cannot rule out the involvement of regulatory mechanisms that affect cell division indirectly through ClpX-mediated (Camberg et al., 2009; Haesser et al., 2009; Männik et al., 2018), or *E. coli* GdiA-like (Lies et al., 2015), analogous systems.

YpsA mutants generated based on the crystal structure and sequence analysis revealed the importance of certain key residues for YpsA function (Figures 1B, 6). Interestingly, G53 and E55 of *B. subtilis* YpsA which form a conserved signature GxD/E motif, are predicted to be important for substrate-binding in the YpsA clade of proteins in the SLOG superfamily (Figure 1B; (Burroughs et al., 2013)). Since foci formation was disrupted in both G53A and E55Q mutants, it is plausible that substrate-binding allows for multimeric complex formation. It is noteworthy that mutants such as G42A and E44Q which are able to form foci, and therefore likely bind substrate, lack the ability to elicit filamentation. Similarly, YpsA-GFP overproducing cells grown in certain growth conditions were able to form foci but unable to induce filamentation (Figure 5). Assuming foci formation is indicative of substrate binding, these observations support a model in which substrate binding by YpsA is a prerequisite for cell division inhibition. However, substrate binding alone is not sufficient to induce filamentation.

The connection between NAD or its derivative ADP-ribose and the members of SLOG superfamily of proteins that belong to YpsA clade has been previously suggested (Burroughs et al., 2015). Given that ADP-ribosylation affects FtsZ polymerization (Ohashi et al., 1999; Ting et al., 2018), and YpsA is in close association with biological conflict systems and phosphoribosyltransferases (Figure 1B), it is possible that YpsA-mediated inhibition of cell division may involve ADP-ribosylation. Similarly, oxidative stress protection provided by YpsA might involve sensing or binding NAD or its derivatives as well. The link between metabolism of nicotinamide nucleotide, glucose availability, and oxidative stress has been reported previously (Inlay and Linn, 1988; Brumaghim et al., 2003).

Lastly, we show that overproduction of YpsA in another Firmicute, *S. aureus*, results in cell enlargement, indicative of cell division inhibition, specifically in a growth rate dependent fashion. This hints at a possible conserved role in cell division for YpsA in these Gram-positive organisms. In *B. subtilis*, a prophage associated protein of unknown function, YoqJ, also belongs to the YpsA family (Figure 1B). Given that there are clear examples of phage proteins affecting bacterial cell division (Zhou and Lutkenhaus, 2005; Ballesteros-Plaza et al., 2013; Kiro et al., 2013; Haesser et al., 2014), it would be interesting to see if YoqJ also influences cell division. Although the GxD/E motif is conserved in YoqJ, several residues we identified to be essential for the cell division function of YpsA are not conserved in YoqJ (Figures 1B, 6). The Firmicutes-specific conserved gene coupling between *ypsA* and *gpsB* starkly contrasts the diversity of the gene neighborhoods found in other branches of the YpsA family phylogenetic tree. Superposition of conserved gene neighborhoods onto the phylogenetic tree (Figure 1A) revealed a stark compartmentalization in conserved genome contexts. The *ypsA* and *gpsB* gene coupling is found only in one of the four major branches in the tree. Each of the other three branches displays distinct conserved neighborhood proclivities including: (1) a branch where YpsA couples strongly in a gene pair relationship with a phosphoribosyltransferase (PRTase) domain, (2) a branch where YpsA is found in scattered associations with various components of NAD processing and salvage pathways, and (3) a diverse collection of contexts across a broad class of bacterial lineages representative of the aforementioned nucleotide-centered biological conflict systems, where YpsA is likely to act in nucleotide signal-generation or nucleotide-sensing (Figure 1A; (Burroughs et al., 2013)). These observations suggest that the *B. subtilis* YpsA may have acquired a more institutionalized role in cell division within the Firmicutes phylum. Nevertheless, understanding the precise biochemical mechanism by which *B. subtilis* YpsA executes its function would potentially shed light on the more general function of YpsA across a wide range of organisms and biological conflict systems.
MATERIALS AND METHODS

Strain Construction and General Methods

All B. subtilis strains used in this study are isogenic derivatives of PY79 (Youngman et al., 1984). See Table S1A for strain information. Overproduction of YpsA was achieved by PCR amplifying ypsA using primer pairs oP106/oP108 (see Table S1B for oligonucleotide information) and ligating the fragment generated cut with SalI and NheI with IPTG-inducible amYE locus integration vector pDR111 (D. Rudner) also cut with SalI for oligonucleotide information) and ligating the fragment generated cut with SalI and NheI with IPTG-inducible amYE locus integration vector pDR111 (D. Rudner) also cut with SalI and NheI and the resulting plasmid was named pGG27. To construct a GFP fusion, ypsA fragment that was amplified with primer pairs oP106/oP107 and digested with SalI and NheI was ligated with gfp fragment generated with oP46/oP24 and cut with Nhel/Sphi and cloned into pDR111 digested with SalI/Sphi, resulting in plasmid pGG28. The G42A, E44Q, W45A, G53A, E55Q, W57A, and W87A mutations were introduced using the QuickChange site-directed mutagenesis kit (Agilent) using pGG28 as template. The strain expressing ypsA::gfp under the control of its native promoter from an ectopic locus was constructed by PCR amplifying ypsA and its promoter region using the primer pairs oP301/oP107 and by digesting the resulting PCR product with HindIII and NheI. The gfp fragment was PCR amplified using the primer pairs oP46/oP47 and digested with Nhel and BamHI. The digested products were then ligated into the amYE integration plasmid pDG1662 cut with HindIII and BamHI, and the resulting plasmid was named pRB43. ypsA-3xflag was constructed via two step PCR using pGG27 as a template. Round one PCR was completed using primers oP106 and oP291. The PCR product from round one was then used as a template for round two PCR, which was completed using primers oP106 and oP292. The final PCR product was then cloned into pDR111 using SalI and Nhel restriction sites, making plasmid pRB33. Similarly, ypsA::gfp-3xflag was constructed via two step PCR using pGG28 as a template. Round one PCR was completed using primers oP106 and oP349. The PCR product from round one was then used as a template for round two PCR, which was completed using primers oP106 and oP350. The final PCR product was then cloned into pDR111 using SalI and Sphi restriction sites, making plasmid pRB34. The engineered plasmids were then used to introduce genes of interest via double crossover homologous recombination into the amYE locus of the B. subtilis chromosome. To produce S. aureus YpsA in S. aureus strain SH1000, ypsA allele (fragment PCR amplified with oRB27/oP314 primer pairs) was cloned into xylese-inducible pEPSA5 plasmid using EcoRI and BamHI restriction sites (Forsyth et al., 2002), generating plasmid pRB36. Plasmids were first introduced into S. aureus RN4220 via electroporation, and then transduced into SH1000 (Eswarani et al., 2018).

Media and Culture Conditions

Overnight B. subtilis cultures grown at 22°C in Luria-Bertani (LB) growth medium were diluted 1:10 into fresh LB medium and grown in 37°C to mid-logarithmic growth phase (OD600 = 0.5), unless otherwise stated. Expression of genes under xylose-controlled promoter was induced by addition of 1% xylose when required.

Sporulation Assay

Sporulation assay was conducted using resuspension protocol as described previously (Eswaramoorthy et al., 2009). Briefly, overnight cultures of B. subtilis cells were grown in LB medium at 22°C, were diluted 1:10 in fresh casein hydrolysate medium (CH, KD Medical) and grown in 37°C to mid-log phase twice before culture was resuspended in Sterlini-Mandelstam sporulation medium (SM, KD Medical) to induce sporulation (Sterlini and Mandelstam, 1969). Growth in CH medium and entry into sporulation in SM medium were monitored via fluorescence microscopy. Total viable cell counts (CFU/ml prior to heat treatment) and spore counts (CFU/ml after incubation at 80°C for 10 min) were obtained for calculating sporulation frequency (spore count/viable count).

Disc Diffusion Assay

All disc diffusion assays were completed on LB agar plates. Strains PY79 and RB42 were grown until OD600 =0.5, and 100 μl of each culture was spread on the surface of LB plates using sterile glass beads. After the plates were dry, 15 μl of 0.1 mM mitomycin C (Alfa Aesar), 1% SDS (Fisher BioReagents), 5 mg/ml daptomycin (Biotang), different concentrations of hydrogen peroxide (Fisher Chemical) was added to 7 mm Whatman filter paper discs, which were then placed equidistant from each other on top of the inoculated plate. Additionally, commercially available discs (Becton-Dickinson) containing 10 units of Penicillin or 30 μg of vancomycin was also used in this assay. Disks containing 15 μl of sterile water were used as our mock control. Plates were then incubated overnight at 37°C. The diameter of the discs (7 mm) was subtracted from the zone of inhibition measurements.

Immunoprecipitation Assay

The YpsA-FLAG immunoprecipitation was performed using FLAGIPT1 immunoprecipitation kit (Sigma-Aldrich) as described previously (Eswaramoorthy et al., 2014). Briefly, 1 ml cell lysates of cells harvested from 20 ml LB culture induced with 250 μM IPTG (final concentration) grown for 2 h post-induction to produce FLAG-tagged proteins or untagged negative control were generated by sonication. Cell extracts were then incubated overnight with 50 μl anti-FLAG M2 affinity beads supplied by the manufacturer. The beads were then washed 3 times with 1x wash buffer and the supernatant was removed by pipetting. Proteins bound to the beads were stripped by adding 80 μl of 2x sample buffer supplied by the manufacturer and heating at 100°C for 5 min. Western blot using anti-Flag antibody (Invitrogen) was used to detect Flag tagged proteins in all fractions collected.
Microscopy
Aliquots containing 1 ml of culture (B. subtilis and S. aureus) were washed in phosphate buffered saline (PBS) and then resuspended in 100 µl of PBS containing 1µg/ml FM-64 (membrane stain). For imaging, 5 µl of sample was then spotted onto a glass bottom dish (MatTek) and it was covered with an 1% agarose pad made with sterile water. Still imaging was completed at room temperature. For time-lapse microscopy, 5 µl aliquots of culture were spotted onto a glass bottom dish (MatTek) and it was covered with an 1% agarose pad made with LB culture medium. Agarose pads were supplemented with 2 µl of FM-64 (1 µg/ml) and/or inducer were required to induce the expression of desired genes prior to data collection. Microscopy was performed using GE Applied Precision DeltaVision Elite deconvolution fluorescence microscope equipped with a Photometrics CoolSnap HQ2 camera and environmental chamber. Typically, 17 planes (Z-stacks) every 200 nm was acquired for time-lapse microscopy to minimize phototoxicity. The images were then deconvolved using SoftWorx software provided by the manufacturer. Fluorescence intensity of GFP signal was quantified using the data inspector tool on the SoftWorx software. Briefly, fluorescence signal was measured in arbitrary units in cells producing GFP and background noise of equivalent area from a neighboring region devoid of cells or cell debris in the field of view was subtracted to acquire the final value.

Sequence Analysis
YpsA sequence similarity searches were performed using the PSI-BLAST program (Altschul et al., 1997) against the non-redundant (NR) database of the National Center for Biotechnology Information (NCBI). Multiple sequence alignments were built by the MUSCLE and KALIGN programs (Edgar, 2004; Lassmann et al., 2009), followed by manual adjustments on the basis of profile–profile and structural alignments.

REFERENCES

copy of this manuscript is available in bioRxiv server (Brzozowski et al., 2018).

SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00623/full#supplementary-material


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Supplemental Data

Deciphering the role of a SLOG superfamily protein YpsA in Gram-positive bacteria

Robert S. Brzozowski¹, Mirella Huber¹, A. Maxwell Burroughs², Gianni Graham¹, Merryck Walker¹, Sameeksha S. Alva¹, L. Aravind², and Prahathees J. Eswara¹

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²National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD 20894, USA

Figure S1. Transcript levels of cotD, ypsA, and gpsB in B. subtilis at various growth conditions (28, 29).

Figure S2. Immunoprecipitation assay. A strain co-producing YpsA-FLAG and YpsA-GFP under the control of IPTG (RB222) was induced at mid-log (OD₆₀₀ =0.5). Cells were harvested 2 h post-induction and processed for anti-Flag immunoprecipitation as described in materials and methods section. Cell lysate (Load) and eluate fractions were subjected to immunoblotting with antisera specific to FLAG, GFP, or SigA.

Figure S3. (A) Growth curves (OD₆₀₀) of strains WT (PY79) and ΔypsA (RB42) grown in LB medium at 37 °C and 22 °C, and in CH medium, followed for 5 h. (B) Growth curves of cells overproducing ypsA (GG82) or ypsA-gfp (GG83) grown in CH medium, with or without the supplementation of 1% glucose or 1% sucrose, monitored for 5 h. (C) Growth curves of strains ypsA (GG82) and ypsA-gfp (GG83) grown in LB medium at 22 °C or 37 °C, tracked for 5 h. All experiments were performed in triplicates and representative graphs are shown for (A), (B), and
(C). (D) Cells (1 ml culture; GG83) harvested at mid-log phase (OD$_{600}$ =0.5) and 3 h post mid-log (stationary) were subjected to immunoblotting against antisera specific to GFP or SigA. Ratio of GFP/SigA (arbitrary units) are shown at the bottom.

**Figure S4.** Cell morphologies of inducible ypsA cells (GG82) grown in the absence (A) or presence (B) of inducer. Also shown are the cell morphologies of inducible ypsA in a strain lacking *ugtP* (RB212) grown in the absence (C) or presence (D) of inducer. Scale bar: 1 μm.

**Table S1.** Strains and oligonucleotides used in this study

**Video S1.** Timelapse of YpsA-GFP foci movement. DIC and GFP fluorescence information of strain GG83 producing YpsA-GFP imaged in the presence of inducer at 1-min interval for 10 min. Arrow indicates foci that move significantly.
Figure S2

Load | Eluate

α-FLAG | YpsA-FLAG
α-GFP | YpsA-GFP
α-SigA | SigA
Figure S3

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GFP/SigA ratio:
- WT vs ΔypsA
- CH medium (ypsA)
- CH medium (ypsA-gfp)
- LB medium
- Mid-log
- Stationary

Graphs showing growth curves and GFP/SigA ratios under different conditions.
Figure S4

-Inducer  +Inducer

YpsA

ugtP

A

B

C

D
### Table S1  Strains and oligonucleotides used in this study

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*BGSC - Bacillus Genetic Stock Center*
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APPENDIX B

Attached Publication 2

Suppressors of YpsA-mediated cell division inhibition in *Bacillus subtilis*

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Keywords: FtsZ; GpsB; filamentation; SLOG; YfhS

Running title: Suppressors of YpsA-induced filamentation in *B. subtilis*

GRAPHICAL ABSTRACT

Isolation of spontaneous suppressors

**GRAPHICAL ABSTRACT**

ABBREVIATED SUMMARY

In *Bacillus subtilis*, we discovered that increased expression of ypsA results in cell division inhibition and impairment of colony formation on solid medium. Colonies that do arise possess compensatory suppressor mutations. Analysis of one such suppressor mutation led us to a protein of unknown function, YfhS, which appears to play a role in regulating cell length and cell width.
SUMMARY

Although many bacterial cell division factors have been uncovered over the years, evidence from recent studies points to the existence of yet to be discovered factors involved in cell division regulation. Thus, it is important to identify factors and conditions that regulate cell division to obtain a better understanding of this fundamental biological process. We recently reported that in the Gram-positive organisms *Bacillus subtilis* and *Staphylococcus aureus*, increased production of YpsA resulted in cell division inhibition.

In this study, we isolated spontaneous suppressor mutations to uncover critical residues of YpsA and the pathways through which YpsA may exert its function. Using this technique, we were able to isolate four unique intragenic suppressor mutations in *ypsA* (E55D, P79L, R111P, G132E) that rendered the mutated YpsA non-toxic upon overproduction. We also isolated an extragenic suppressor mutation in *yfhS*, a gene that encodes a protein of unknown function. Subsequent analysis confirmed that cells lacking *yfhS* were unable to undergo filamentation in response to YpsA overproduction. We also serendipitously discovered that YfhS may play a role in cell size regulation.

INTRODUCTION

Bacterial cell division is an essential process orchestrated by a multitude of cell division proteins (Haeusser & Margolin, 2016). During growth an essential cell division protein FtsZ, forms a ring-like structure and marks the site of division. There it serves in the recruitment of additional divisome proteins and commences septation (Du & Lutkenhaus, 2017, Errington & Wu, 2017). Although known FtsZ regulatory systems, such as the Min system and nucleoid occlusion, have been well characterized (Eswara

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recent studies have determined that correct cell division site selection can occur independent of these mechanisms in both *Bacillus subtilis* and *Escherichia coli* (Rodrigues & Harry, 2012, Bailey *et al.*, 2014). These findings highlight the need to investigate and discover other factors involved in regulating cell division in bacteria. In our lab, we have identified a potential cell division regulator in *B. subtilis* and *Staphylococcus aureus*, YpsA (Brzozowski *et al.*, 2019a).

YpsA is conserved in the Firmicutes phylum of Gram-positive bacteria and appears to be in a syntenous relationship with a known cell division protein, GpsB (Brzozowski *et al.*, 2019a). The crystal structure of *B. subtilis* YpsA was solved by a structural genomics group in 2006 [PDB ID: 2NX2; (Ramagopal *et al.*, 2006)]. Based on the structural features, YpsA was placed as the founding member of the “YpsA proper” subclade within the SLOG (SMF/DprA/LOG) protein superfamily (Burroughs *et al.*, 2015), yet the precise function of YpsA remains to be elucidated. The structure of YpsA resembles that of DprA (RMSD with DprA of *Helicobacter pylori* is 2.79 Å, PDB ID: 4LJR), another member of the SLOG superfamily, which is a single-stranded DNA-binding protein involved in DNA recombination (Yadav *et al.*, 2014, Wang *et al.*, 2014). Previously we had found that YpsA provides oxidative stress protection in *B. subtilis* and that overproduction of YpsA results in cell division inhibition, through FtsZ mislocalization, in a growth rate dependent manner (Brzozowski *et al.*, 2019a). Additionally, using site-directed mutagenesis we identified multiple amino acid residues that are potentially important for the structure and/or function of YpsA, including residues located in the conserved substrate binding pocket made up of glycine and...
glutamate residues predicted by Burroughs et al., 2015 (Burroughs et al., 2015). In addition, we have shown that the function of YpsA in cell division is also conserved in the Gram-positive pathogen, S. aureus (Brzozowski et al., 2019a).

In this study we utilized a classic spontaneous suppressor isolation technique for further identification of critical amino acid residues that are important for YpsA structure and/or function, and for the elucidation of the molecular mechanism through which YpsA acts. By screening for suppressor mutations of a lethal YpsA overproduction phenotype, we were able to isolate and characterize four unique intragenic suppressor mutations (E55D, P79L, R111P, G132E). Each of these mutations was found to prevent lethality and related cell division inhibition. In addition, we also identified an extragenic suppressor mutation that introduced a premature stop codon in the yfhS gene, which codes for a protein of unknown function. Upon subsequent analysis, we verified that in cells lacking yfhS, YpsA-dependent cell division inhibition is abolished. Here, we speculate the possible ways by which YfhS and YpsA-mediated cell division phenotypes could be linked. Interestingly, during the course of our experiments, we discovered that YfhS may play a role in cell size regulation, as yfhS null cells are significantly smaller in cell width and length when compared to the wild type control.

RESULTS

Overexpression of ypsA results in a growth defect on solid medium

We have previously shown that overproduction of either YpsA or YpsA-GFP results in severe filamentation in B. subtilis [(Brzozowski et al., 2019a); Fig. 1A]], a phenotype
that is characteristic of cell division inhibition in this organism. To test whether filamentous growth in the presence of inducer results in a distinguishable phenotype on solid medium, we conducted a spot assay. Briefly, serial dilutions of exponentially growing wild type (WT) cells, and cells containing an IPTG-inducible copy of either *ypsA* or *ypsA-gfp* were spotted on solid growth medium with or without inducer. In the absence of inducer all strains of all tested dilutions grew similar to the WT control (Fig. 1B; see left panel). In the presence of inducer, we observed a significant growth defect associated with both YpsA and YpsA-GFP overproduction, suggesting that cell division inhibition caused by *ypsA* or *ypsA-gfp* overexpression was lethal (Fig. 1B; see right panel). Interestingly, YpsA-GFP overproduction resulted in a more severe growth phenotype compared to untagged YpsA. To test whether this difference is due to increased accumulation of YpsA-GFP in the cells, we tagged both YpsA and YpsA-GFP with a FLAG tag at their C-terminus and conducted an anti-FLAG western blot analysis. Overproduction of YpsA-FLAG and YpsA-GFP-FLAG resulted in filamentation and a growth defect on solid medium that was of similar extent when compared to their non-FLAG tagged counterparts (Figs. S1A and S1B). The ratio of YpsA-GFP-FLAG and SigA (internal loading control) was similar to that of YpsA-FLAG and SigA (0.68 and 0.80 respectively; Fig. S1C), suggesting that the increased lethality of the GFP-tagged version is not due to any changes in accumulation. Next, we utilized the severe lethality elicited by YpsA-GFP overproduction as a tool to isolate spontaneous suppressors.

**Isolation of spontaneous suppressor mutations**
The YpsA-GFP overproducing strain was streaked out for single colony isolation on multiple inducer containing plates and the plates were incubated overnight as described in the methods section. Only a few colonies formed per plate, presumably due to spontaneous suppressor mutations, which allow for normal growth despite the presence of inducer. After multiple iterations of suppressor isolation, likely mutations were subsequently determined to be either intragenic (within inducible \textit{ypsA-gfp}, henceforth noted as \textit{ypsA*-gfp} for simplicity) or extragenic (elsewhere on the chromosome), and their chromosomes were sequenced to identify the mutations (Fig. S2). Using this approach, we were able to isolate four unique intragenic suppressor mutations: G132E, P79L, R111P, and E55D (listed in the order of isolation). Immunoblotting indicated that these mutant versions of YpsA were stably produced (Fig. 2L). Next, fluorescence microscopy was used to determine whether these mutations were able to rescue the lethal filamentous phenotype observed when unmutated YpsA-GFP was overproduced. This was carried out on exponentially growing cells of the \textit{ypsA-gfp} overexpression strain and all \textit{ypsA*-gfp} intragenic suppressor strains in the absence or presence of inducer. In the absence of inducer all strains exhibited similar cell lengths [YpsA-GFP: 3.23 ± 0.74 μm (Fig. 2A); G132E: 3.49 ± 0.93 μm (Fig. 2C); P79L: 3.24 ± 0.81 μm (Fig. 2E); R111P: 3.16 ± 0.76 μm (Fig. 2G); E55D: 3.23 ± 0.83 μm (Fig. 2I) | n=100 for all cell length measurements]. Upon the addition of inducer, we found that cells overexpressing \textit{ypsA*-gfp} did not exhibit filamentation, unlike the \textit{ypsA-gfp} control [YpsA-GFP: 8.93 ± 5.67 μm (Fig. 2B); G132E: 2.99 ± 0.88 μm (Fig. 2D); P79L: 3.29 ± 0.81 μm (Fig. 2F); R111P: 3.06 ± 0.98 μm (Fig. 2H); E55D: 3.10 ± 0.83 μm (Fig. 2J)], indicating that the intragenic suppressors were unable to elicit filamentation upon overproduction. We also
noted that G132E, P79L, and R111P suppressors displayed impaired foci formation in comparison to the \textit{ypsA-gfp} control (Figs. 2B, 2D, 2F, and 2H). However, foci formation in E55D suppressor was not impaired (Fig. 2J).

Each of the intragenic suppressors were subjected to a spot assay to test whether these point mutations were able to grow normally in contrast to the \textit{ypsA-gfp} overexpression strain that displayed a lethal phenotype on solid medium in the presence of inducer. In the absence of inducer all strains grew similar to the WT control (Fig. 2K; see top panel). When grown in the presence of inducer, overexpression of \textit{ypsA-gfp} resulted in a severe growth defect (Fig. 2K; see bottom panel). However, growth was similar to WT in all intragenic suppressor strains when grown in the presence of inducer (Fig. 2K; see bottom panel). Given that these mutants were unable to cause filamentation, it appears that the lethality is directly linked to the ability of YpsA to elicit filamentation. Collectively, these data indicate that the residues E55, P79, R111, and G132 are critical for the function of YpsA, especially in regard to cell division inhibition.

\textbf{Structural analysis of the intragenic suppressor mutations}

Three of the four YpsA mutants have residues that are buried in the core: E55D, G132E, and P79L (Fig. 3A). When large mutations occur in this environment, misfolding and loss of function is often the consequence (Baruah & Biswas, 2014). Amongst these mutants, P79 is significant because it, and its adjacent residue F80, are strictly conserved amongst the YpsA clade of Firmicutes (Burroughs \textit{et al.}, 2015, Brzozowski \textit{et al.}, 2019a). The crystal structure of \textit{B. subtilis} YpsA (PDB ID: 2NX2) reveals P79 and
F80 also line the possible DNA binding groove of YpsA (Ramagopal et al., 2006). The positioning of an aromatic side chain here suggests it may facilitate DNA binding by forming stacking interactions with nucleobases (Baker & Grant, 2007). The P79L mutation not only creates severe clashes with surrounding residues, but likely perturbs the positioning of F80, and therefore may directly impair DNA or nucleotide binding (Fig. 3B). Likewise, the E55D mutant affects a second, highly conserved segment of the putative DNA binding domain, the GxE motif. In YpsA, E55 is highly coordinated by five potential hydrogen bonds with the side chain and backbone of S49, the T7 sidechain, and the backbone amide of Q51 (Fig. 3C). Considering the size and physicochemical properties of their sidechains, one would expect an E\rightarrow D mutation to have a non-deleterious effect on YpsA. However, in this instance, shortening the sidechain of E55 by a methylene results in the weakening or total loss of these hydrogen bonds and likely destabilizes the possible DNA binding groove. In addition, the aliphatic part of the E55 side chain forms extensive van der Waals interactions with nearby residues such as Q51 and others, and the carboxylate group of an aspartate residue at this position would also clash with these surrounding residues. The third core mutation, G132E, is located at the beginning of a β-strand and is surrounded by multiple bulky and hydrophobic residues including Y164, P162, L1, L4, and F38 (Fig. 3D). Conversion from glycine to any other residue besides alanine results in clashes that will affect the secondary structural elements from which the surrounding residues originate. Indeed, every possible G132E rotamer produces significant clashes, with interatomic distances less than 2.2 Å.
The R111P mutation is the only intragenic suppressor mutation that involves a solvent exposed residue. Here R111 normally forms a salt bridge with the neighboring E114 (Fig. 3E). The conversion from a positively charged side chain to a nonpolar one eliminates this interaction. Furthermore, the cyclic nature of the proline side chain introduces a steric clash with the backbone amide nitrogen of its adjacent residue, H110. The R111P mutation will likely force conformational changes in the protein backbone and cause significant disruptions in intramolecular interactions involving nearby residues. A second scenario that leads to the disruption of YpsA function in this mutant involves the impairment of intermolecular interactions and macromolecular recognition. It is possible that a mutation from R→P prevents interaction with other protein partners of YpsA.

Isolation and validation of an extragenic suppressor mutation in yfhS

Using the same suppressor screening approach (Fig. S2), we were able to isolate and validate an extragenic suppressor mutation, which is a duplication of a stretch of 10 nucleotides that introduces a premature stop codon in yfhS (Fig. S3A). YfhS is a 74 amino acid containing protein of unknown function. yfhS is annotated as a sporulation gene upregulated by SigE sigma factor during sporulation (Zhu & Stulke, 2018). However, there is no sporulation defect in a yfhS null strain (Yamamoto et al., 1999). yfhS may also be regulated by the transcription factor AbaA (Banse et al., 2008).

To test whether disruption of yfhS restores normal cell length in cells overexpressing either ypsA or ypsA-gfp, we generated a strain harboring an inducible copy of either
ypsA or ypsA-gfp in a yfhS null background. These strains were then screened with the appropriate controls via a spot assay in order to observe whether or not the yfhS deletion was able to restore normal growth on solid medium even when YpsA or YpsA-GFP was overproduced. In the absence of inducer, WT cells and cells containing an inducible copy of either ypsA or ypsA-gfp grew similarly (Fig. 4A). Cells lacking yfhS formed small colonies in comparison to WT suggesting an intrinsic growth phenotype associated with the deletion of yfhS. Cells harboring an inducible copy of ypsA or ypsA-gfp in a yfhS null background grew similar to the yfhS null control strain. When grown in the presence of inducer, as shown in Fig. 1B, cells harboring an inducible copy of ypsA showed a moderate growth defect while inducible ypsA-gfp strain exhibited a severe growth defect (Fig. 4B). In the presence of inducer, cells harboring a yfhS knockout and cells harboring a yfhS knockout with an inducible copy of either ypsA or ypsA-gfp grew similarly, suggesting that deletion of yfhS prevents elicitation of lethal phenotypes displayed by YpsA and YpsA-GFP overproducing cells (Fig. 4B). To ensure the phenotype was specific to the disruption of the native copy of yfhS, we introduced yfhS at an ectopic locus under an inducible promoter. In this complementation strain, the presence of inducer or even leaky expression in the absence of inducer, restored WT-like growth (Fig. 4A). Interestingly, the defective growth phenotype of YpsA and YpsA-GFP overproducing cells was also restored in the presence of inducer in the complementation strain (compare Figs. 4A and 4B).

Next, we inspected the cell morphology of all strains tested in Figs. 4A and 4B through fluorescence microscopy. Cell division in cells harboring an inducible copy of either
ypsA or ypsA-gfp, but not in WT control, were inhibited upon the addition of inducer (Fig. 4C-H), as discussed previously [Fig. 1A; (Brzozowski et al., 2019a)]. The quantification of cell lengths are shown in Fig. 4U. The cell lengths of the WT control strain in the absence and presence of inducer were similar [WT (- inducer): 2.72 ± 0.68 μm (Fig. 4C); WT (+ inducer): 2.77 ± 0.69 μm (Fig. 4D)]. On the contrary, as expected, cells overproducing YpsA or YpsA-GFP exhibited filamentation in the presence of inducer [YpsA (- inducer): 2.59 ± 0.71 μm (Fig 4E); YpsA (+ inducer): 6.36 ± 2.56 μm (Fig 4F)]; YpsA-GFP (- inducer): 2.99 ± 0.73 μm (Fig. 4G); YpsA-GFP (+ inducer): 6.77 ± 3.48 μm (Fig. 4H)].

Upon imaging the ΔyfhS cells, to our astonishment, we noticed that the average cell length was smaller than WT cells [ΔyfhS (- inducer): 1.99 ± 0.61 μm], compare Figs. 4I and 4C. In addition to a smaller cell length, the average cell width of ΔyfhS cells also appeared to be smaller when compared to WT [WT (- inducer): 0.78 ± 0.06 μm (Fig. 4C); ΔyfhS (- inducer): 0.67 ± 0.08 μm (Fig. 4I)] n=100. This observation hints at the possible role for YfhS in cell size regulation either directly or indirectly. Addition of inducer had no effect on the average cell length of cells lacking yfhS [ΔyfhS (+ inducer): 1.91 ± 0.48 μm; Fig. 4J]. When cells containing an inducible copy of either ypsA or ypsA-gfp in a yfhS null background were imaged, they also exhibited smaller cell lengths in the absence of inducer [ΔyfhS + YpsA (- inducer): 1.93 ± 0.52 μm (Fig. 4K); ΔyfhS + YpsA-GFP (- inducer): 2.02 ± 0.55 μm (Fig. 4M)], suggesting that that small-cell phenotype is intrinsically linked to the lack of the yfhS gene. Intriguingly, overproduction of either YpsA or YpsA-GFP did not result in filamentation in a yfhS null
background \( \Delta yfhs + YpsA \) (+ inducer): 1.97 ± 0.56 \( \mu m \) (Fig. 4L); \( \Delta yfhs + YpsA\)-GFP (+ inducer): 2.17 ± 0.72 \( \mu m \) (Fig. 4N), indicating that YpsA-mediated cell division inhibition is dependent on YfhS. The ratio of YpsA-GFP-FLAG and SigA in the WT background and \( yfhs \) null background were similar (0.68 and 0.56 respectively; Fig. S1C), suggesting that the elimination of cell division inhibition is not due to defective accumulation of YpsA-GFP. We did observe a 2-fold increase in the ratio of YpsA-FLAG and SigA between WT and \( \Delta yfhs \) background (0.80 and 1.59 respectively; Fig. S1C).

However, this can be attributed to lower levels of SigA that we have seen reproducibly in this strain background, when the optical density is standardized between the strains tested. Thus, we conclude that the abolition of cell division inhibition in \( yfhs \) null strain is not due to defective accumulation of YpsA or YpsA-GFP.

We further confirmed that the \( yfhs \) deletion phenotype is linked specifically to \( yfhs \), and not due to any kind of polar effect, by using the complementation strain described earlier. Fluorescence microscopy revealed that the characteristic small-cell phenotype of \( \Delta yfhs \) was no longer observed, even in the absence of inducer, likely due to the leaky expression of \( yfhs \) in the complementation strain [\( \Delta yfhs + yfhs \) (- inducer): 2.23 ± 0.58 \( \mu m \) (Fig. 4O)]. When the expression of ectopic \( yfhs \) was induced by the addition of inducer, the average cell length resembled that of the WT control [\( \Delta yfhs + yfhs \) (+ inducer): 2.47 ± 0.65 \( \mu m \)], compare Figs. 4P and 4D. As expected, cells carrying an IPTG-inducible copy of \( ypsA \) or \( ypsA\)-gfp in the complementation strain background, in the absence of inducer, appeared similar to the WT control [\( \Delta yfhs + yfhs + YpsA \) (- inducer): 2.31 ± 0.76 \( \mu m \) (Fig. 4Q); \( \Delta yfhs + yfhs + YpsA\)-GFP (- inducer): 2.44 ± 0.80]
μm (Fig. 4S)]. However, in the presence of inducer filamentation was restored in these two strains \( \Delta yfhS + yfhS + YpsA (+ \text{ inducer}) \): 5.60 ± 2.47 μm (Fig. 4R); \( \Delta yfhS + yfhS + YpsA\text{-GFP} (+ \text{ inducer}) \): 7.25 ± 3.64 μm (Fig. 4T), confirming that YpsA-mediated filamentation requires YfhS. The precise reason for this requirement is unclear at this time.

**DISCUSSION**

Although many factors involved in facilitating the cell division process have been discovered in *B. subtilis* (Errington & Wu, 2017) and *E. coli* (Du & Lutkenhaus, 2017), our understanding is still incomplete even in these model organisms as evidence of yet to be uncovered factors exists (Rodrigues & Harry, 2012, Bailey *et al.*, 2014). We reported previously that YpsA is such a factor, which appears to play a role in cell division in *B. subtilis* and *S. aureus* (Brzozowski *et al.*, 2019a). The precise mechanism by which YpsA functions remains unclear. The structure of YpsA and another SLOG superfamily member DprA, a single-stranded DNA binding protein, is similar. Thus it is possible YpsA also binds DNA, or nucleotides such as NAD or ADP-ribose as speculated previously (Brzozowski *et al.*, 2019a). We undertook this study to shed light on the possible pathways through which YpsA functions. In this report, we describe our observations of YpsA-mediated lethality on solid medium and utilized that phenomenon as a tool to isolate spontaneous suppressors. Using this technique, we have isolated intragenic suppressors and an extragenic suppressor that abolishes YpsA-mediated toxicity.
We have isolated four intragenic suppressors (E55D, P79L, R111P, G132E) using our screen. Given that E55 and P79 residues are highly conserved among YpsA, perhaps not surprisingly, mutations in those residues render YpsA inactive at least with respect to its function in cell division. It appears that in the E55D mutation, even though it retains the negative charge, shortening of the side chain appears to weaken the ability to form hydrogen bonds with neighboring residues. The mutations in highly conserved P79 and weakly conserved G132 residues create several steric clashes, which explains why the function of YpsA in cell division is affected. The mutation in the solvent exposed residue R111 may result in weakened intramolecular and/or intermolecular interactions. Thus, our screen has identified multiple key residues that are essential for the proper function of YpsA in regard to cell division inhibition.

The extragenic suppressor mutation we isolated introduced a premature stop codon in the yfhS open reading frame. YfhS is a relatively small protein (74 amino acids) of unknown function. yfhS is upregulated during sporulation through the SigE transcription factor (Yamamoto et al., 1999) and possibly by AbbA (Banse et al., 2008), thus it has been classified as a sporulation gene. In our results we note that ΔyfhS cells appear smaller in both width and length compared to our WT control, suggesting that YfhS may have a role in cell size regulation during vegetative growth. Furthermore, we tested and confirmed that YpsA-mediated cell division inhibition is dependent on the presence of full length YfhS.
Given that YfhS is a protein of unknown function, how YfhS and YpsA are linked remains to be determined. During our course of experiments, we noticed that \( \Delta yfhS \) cells grew slower than our WT control (Fig. S3B). We have previously shown that YpsA-mediated cell division inhibition is a growth rate-dependent phenomenon (Brzozowski et al., 2019a), thus it is possible that the abolition of cell division inhibition in cells lacking \( yfhS \) could be attributed to slow growth. However, at this point we cannot rule out the possibility of a direct mechanistic link between YpsA and YfhS, since YpsA plays a role in cell division and YfhS appears to play a role in cell size regulation.

**EXPERIMENTAL PROCEDURES**

**Strain construction and general methods**

All *B. subtilis* strains utilized during the course of this study are derivatives of the laboratory strain PY79 (Youngman et al., 1984). Table S1 contains all relevant strain and oligonucleotide information. The construction of strains overexpressing *ypsA*, *ypsA-gfp*, *ypsA-flag*, and *ypsA-gfp-flag* have been described previously (Brzozowski et al., 2019a). In order to construct a *B. subtilis* strain containing an inducible copy of *yfhS*, *yfhS* was PCR amplified from PY79 chromosomal DNA using primer pair oRB59/oRB60. The resulting PCR product was digested with SalI and NheI restriction enzymes and cloned into pDR111 (D. Rudner), also digested with SalI and NheI, to construct plasmid pRB54. The constructed plasmids were then transformed into competent PY79 cells to introduce genes of interest via double crossover homologous recombination into either the native and non-essential *amyE* locus or into a second *amyE* locus (bkdB::Tn917::amyE::cat; Amy Camp).
Media and culture conditions

Overnight *B. subtilis* cultures were grown at 22 °C in Luria-Bertani (LB) growth medium, and subsequently diluted 1:10 into fresh LB medium. Cultures were grown at 37 °C in a shaking incubator to mid-logarithmic growth phase (OD$_{600}$=0.5), unless otherwise stated. In order to induce the expression of genes under the control of an IPTG-inducible promoter, 250 μM IPTG was added to growing cultures, where required, at mid-logarithmic phase, unless stated otherwise.

Spot assay

All spot assays were completed on LB agar plates supplemented with 1mM IPTG, where required, to induce the expression of genes under the control of an IPTG-inducible promoter. Required strains were first grown to mid-logarithmic phase (OD$_{600}$=0.5) at 37°C while shaking, and subsequently standardized to an OD$_{600}$=0.1. Following standardization, serial dilutions of each of the strains were spotted onto the appropriate LB plates at a volume of 1 μl. Plates were incubated overnight (approximately 14 hours) at 37 °C. On the following day, plates were observed for growth defects.

Isolation of spontaneous suppressor mutations

The severe growth defect associated with the strain overproducing YpsA-GFP (GG83) allowed for the isolation of spontaneous suppressor mutations that were able to restore growth similar to the WT control. Suppressor mutations were isolated and determined to
be either intragenic or extragenic as indicated in Fig. S2. For this purpose, the strain GG83 was plated on LB agar plates containing 1 mM IPTG to induce the expression of *ypsA-gfp*, and plates were incubated overnight at 37 °C. After overnight incubation, plates were examined for growth defects associated with the overproduction of YpsA-GFP. PY79 was utilized as a control to ensure that any reduction in growth was specifically due to the overproduction of YpsA-GFP. Single colonies of GG83 that did arise in the presence of inducer (likely containing suppressor mutations) were isolated from the original plate and used to inoculate new LB agar plates that were then grown overnight at 37 °C. Genomic DNA was then isolated from each of the strains containing suppressor mutations using standard phenol-chloroform DNA extractions. Isolated genomic DNA was then used to transform WT PY79 cells, which were then screened for integration of *ypsA-gfp* into the non-essential *amyE* locus. The resulting transformants were then inoculated onto LB agar plates supplemented with 1 mM IPTG to induce the expression of *ypsA-gfp*, and plates were incubated overnight at 37 °C. On the following day plates were screened for growth defects associated with *ypsA-gfp* overexpression. PY79 was used as a control to ensure that any observed growth defect was specifically due to the production of YpsA-GFP. If strains harboring an inducible copy of *ypsA-gfp* isolated during our suppressor screen were now able to grow in the presence of the IPTG inducer, then the suppressor mutations were noted as possibly intragenic (*ypsA*-gfp). If the strains were still unable to grow in the presence of the IPTG inducer, then the mutations were labeled as possibly extragenic - as this indicated that the inducible copy of *ypsA-gfp* within the *amyE* locus did not contain any mutations that were able to restore WT-like growth. All strains determined to contain intragenic suppressor
mutations within the IPTG-inducible copy of ypsA-gfp at the amyE locus were screened via fluorescence microscopy to ensure GFP fluorescence, ruling out some potential mutations within the promoter region, frame-shift mutations, and introduction of premature stop codons. Genomic DNA was isolated from each of the ypsA*-gfp strains containing intragenic suppressor mutations and was then used as a template for PCR using primer pair oP106/oP24 to amplify the ypsA-gfp within the amyE locus. The resulting PCR products were sequenced using a 3’ internal GFP sequencing primer (oP212) by GENEWIZ (South Plainfield, NJ). Sequence analysis was completed using ApE Plasmid Editor (v2.0.51) (M. Wayne Davis), and multiple sequence alignments were built using Clustal Omega multiple sequence alignment software (Sievers et al., 2011). All strains characterized as containing extragenic suppressor mutations that restored WT-like growth to strains overproducing YpsA-GFP were subjected to additional screening prior to whole genome sequencing by integrating a new copy of ypsA-gfp into the amyE locus. The original copy of ypsA-gfp was first replaced by a chloramphenicol resistance cassette, and the resulting strain was then transformed with pGG28 (Brzozowski et al., 2019a), to reintroduce a new copy of ypsA-gfp into the amyE locus. The resulting strains were then used to inoculate LB agar plates supplemented with 1 mM IPTG to verify that they were still able to grow in the presence of inducer unlike the GG83 parental strain. PY79 and GG83 were used as controls on these plates. Plates were incubated overnight at 37 °C and observed for any growth defects associated with YpsA-GFP overproduction on the following day. Genomic DNA was isolated from strains containing extragenic suppressor mutations using the Wizard
Genomic DNA Purification Kit (Promega) and sent for whole genome sequencing (Tufts University School of Medicine Genomics Core).

**Bioinformatics and variant detection**

Data was analyzed using CLC Genomics Workbench 11 (Qiagen Bioinformatics). First, raw reads were aligned to the PY79 reference sequence (CP00681) using the Map Reads to Reference tool. Output read mappings were then subject to coverage analysis and variant detection. The Basic Variant Detection tool was used to generate a variant track and variant table output in consideration with coverage results. Resultant amino acid changes of variants unique to extragenic suppressors were examined using the Amino Acid Changes tool (version 2.4) using set for genetic code parameter 11: Bacterial, Archaeal, and Plant Plasmid. Subsequently, suppressor mutations were also verified manually.

**Structural analysis**

All figures and rotamers were generated using PyMOL (Schrödinger, LLC).

**Growth Curves**

PY79, RB314, and RB409 were first grown to mid-logarithmic phase (OD$_{600}$=0.5) in LB broth at 37 °C with shaking and subsequently standardized to an OD$_{600}$=0.1. IPTG was added to the growth medium at a final concentration of 1 mM, where required to induce the expression of genes of interest. Cultures were then grown in LB medium at 37 °C.
with shaking for a total elapsed time of 6 h. Growth curves were plotted using GraphPad Prism version 8.3.1 (GraphPad Software, La Jolla, California, USA).

**Microscopy**

Microscopy was completed by taking 1 ml aliquots of *B. subtilis* cultures and washing with 1X phosphate buffered saline (PBS) through centrifugation. Cells were then resuspended in 100 μl of PBS, and the red membrane stain FM4-64 was added at a final concentration of 1 μg/ml. The sample was prepared for microscopy by spotting 5 μl of the cell suspension onto the glass coverslip of a MatTek glass bottom dish and subsequently covering it with a 1% agarose pad made with sterile water as described previously (Brzozowski *et al.*, 2019b). All imaging was completed at room temperature inside of an environmental chamber using a GE Applied Precision DeltaVision Elite deconvolution fluorescence microscope. Photos were taken using a Photometrics CoolSnap HQ2 camera. All images were acquired by taking 17 Z-stacks at 200 nm intervals. Images were deconvolved through the SoftWorx imaging software provided by the microscope manufacturer.

**Immunoblot Analysis**

*B. subtilis* strains were grown overnight at 22 °C in LB growth medium, and then diluted 1:10 into fresh LB the following day. Cultures were grown to an OD$_{600}$=0.5 and subsequently induced with 1 mM IPTG where required to induce the expression of the genes of interest. Cultures were then grown to OD$_{600}$=1.0 and following the induction period, 1 ml aliquots of cultures were centrifuged, and cell lysis was completed by
resuspending the cell pellet in a protoplast buffer containing 0.5 M sucrose, 20 mM MgCl₂, 10 mM KH₂PO₄, and 0.1 mg/ml lysozyme. Samples were incubated at 37°C for 30 min and then prepared for SDS-PAGE. Following electrophoresis, samples were transferred onto nitrocellulose membrane and subsequently probed with antibodies against GFP, FLAG (Proteintech Group Inc.), or B. subtilis SigA (M. Fujita), which was used as an internal loading control.

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AUTHOR CONTRIBUTIONS
The conception and design of the study (RSB, PJE), data acquisition (RSB, JJC, ANA, PJE), analysis and/or interpretation of the data (RSB, BRT, MDS, YC, LNS, PJE), and writing of the manuscript (RSB, MDS, YC, PJE).

FIGURE LEGENDS
Figure 1. Overproduction of YpsA or YpsA-GFP results in a lethal phenotype. (A) Cell morphology of wild type (PY79; WT) cells and cells harboring an IPTG-inducible copy of either ypsA (GG82) or ypsA-gfp (GG83) grown in the absence of inducer or in the presence of 250 μM IPTG. Fluorescence of red membrane stain (FM4-64; red) and GFP
(green) are shown. Scale bar: 1 μm. (B) Spot assays of WT cells (PY79) and cells containing an inducible copy of either ypsA (GG82) or ypsA-gfp (GG83). Serially diluted standardized cultures were spotted on plates containing no inducer (left panel) or 1 mM IPTG (right panel) and grown overnight at 37 ºC. Corresponding dilution factors are indicated below.

**Figure 2.** Isolation of spontaneous suppressors. (A-J) Fluorescence microscopy comparing cells containing an inducible copy ypsA-gfp (GG83) and cells containing intragenic mutations (ypsA*-gfp) isolated during the suppressor screen that resulted in single amino acid changes: G132E (RB300), P79L (RB301), R111P (RB328), and E55D (RB327). Cells were grown in the absence (A, C, E, G, I) or in the presence (B, D, F, H, J) of 250 μM IPTG. Fluorescence of FM4-64 (red) and GFP (green) are shown. Scale bar: 1 μm. (K) Spot assays of strains harboring an IPTG inducible copy of ypsA-gfp (GG83) or ypsA*-gfp (RB300, RB301, RB328, RB327) grown without inducer (top panel) or with 1 mM IPTG (bottom panel). Corresponding dilution factors are shown on top. (L) Stability of YpsA-GFP and YpsA*-GFP variants were confirmed when cells were grown in the presence of inducer. Cell lysates were probed via immunoblotting using anti-GFP and anti-SigA (loading control) antisera.

**Figure 3.** Structural analysis of the intragenic suppressors. (A) Crystal structure of *B. subtilis* YpsA (PDB ID: 2NX2) with sites of mutation colored in orange. (B-E) Computationally generated mutants are shown in yellow. Hydrogen bonds are shown as black dashes, while steric clashes are represented as red dashes. For clarity, only the
most severe clashes are indicated with interatomic distances less than 2.2 Å. (B) The P79L mutation generates severe clashes with multiple surrounding residues. (C) The E55D mutation in the putative DNA-binding groove results in the potential loss of five hydrogen bonds, destabilizing this region. (D) The G132E mutation, similar to P79L mutant, involves a core residue that cannot accommodate any large side chains without severe steric clashes. (E) The R111P mutant eliminates a salt bridge with E114 and produces a clash with the adjacent H110 backbone. As a surface residue, it also potentially disrupts intermolecular interactions and signaling.

**Figure 4.** Deletion of yfhS rescues YpsA-mediated toxicity and associated filamentation. (A-B) Spot assay of WT cells (PY79), ΔyfhS cells (RB314), ΔyfhS + yfhS (RB409) cells, and cells overexpressing either ypsA or ypsA-gfp in an otherwise wild type background (GG82 and GG83), a ΔyfhS background (RB288 and RB289), or in a ΔyfhS complementation strain where an intact copy of yfhS is engineered to be under the control of an IPTG-inducible promoter at an ectopic locus (RB410 and RB411). Cultures were standardized and serial dilutions were spotted on solid medium without inducer (A) or with 1 mM IPTG (B). Corresponding dilution factors are indicated on top. (C-T) Fluorescence microscopy comparing cell morphologies of WT cells (PY79), ΔyfhS cells (RB314), ΔyfhS + yfhS (RB409) cells, and cells overexpressing either ypsA or ypsA-gfp in an otherwise wild type background (GG82 and GG83), a ΔyfhS background (RB288 and RB289), or in a ΔyfhS complementation strain (RB410 and RB411). Cells were imaged in the absence of inducer (C, E, G, I, K, M, O, Q, S) or in the presence of 250 μM IPTG (D, F, H, J, L, N, P, R, T). Fluorescence signal of FM4-64 membrane dye (red).
and GFP (green) are shown. Scale bar: 1 μm. (U) Cell lengths of strains shown in panels C-T were quantified. The corresponding mean value and standard deviations (n=100) are shown.

Table S1. Strains and oligonucleotides used in this study.

Figure S1. Analysis of YpsA accumulation. (A) Fluorescence microscopy of strains containing an IPTG-inducible copy of either ypsA-flag (RB121) or ypsA-gfp-flag (RB125) grown in the absence of inducer (left panels) or in the presence of 250 μM IPTG (right panels). Fluorescence of FM4-64 membrane dye (red) and GFP (green) are shown. Scale bar: 1 μm. (B) Spot assays including wild-type cells (PY79) and cells containing an IPTG-inducible copy of either ypsA (GG82), ypsA-gfp (GG83), ypsA-flag (RB121), or ypsA-gfp-flag (RB125). Dilutions of standardized cultures were spotted on solid medium without inducer (left panel) or containing 1 mM IPTG (right panel). Corresponding dilution factors are indicated below. (C) Anti-FLAG and anti-SigA (loading control) immunoblots of RB121 (YpsA-FLAG), RB412 (∆yfhS + YpsA-FLAG), RB125 (YpsA-GFP-FLAG), and RB413 (∆yfhS + YpsA-GFP-FLAG) cell lysates. The FLAG/SigA ratios corresponding to each lane are shown.

Figure S2. Flow chart detailing the methodology used to screen spontaneous suppressor mutations.

Figure S3. Analysis of yfhS extragenic suppressor mutation. (A) Pairwise alignment of the yfhS sequence in WT (PY79) and the extragenic suppressor (RBSS6E11). The
source of 10-nucleotide duplication is highlighted. (B) Growth curves of WT (PY79),
\( \Delta yfhS \) (RB314), and \( yfhS \) complementation strain (RB409) are shown.

REFERENCES


Figure 1
Figure 2

- Inducer
  + Inducer

A YpsA-GFP
B
C G132E
D
E P79L
F
G R111P
H
I E55D
J

K WT
L YpsA-GFP
M G132E
N P79L
O R111P
P E55D
Q

WT
YpsA-GFP
G132E
P79L
R111P
E55D

D -GFP
D -SigA

YpsA-GFP
G132E
P79L
R111P
E55D

α-GFP
α-SigA
Figure 3
Figure 4

- Inducer

+ Inducer

WT
YpsA
YpsA-GFP
ΔyfhS
YpsA
YpsA-GFP
ΔyfhS
YpsA
YpsA-GFP
ΔyfhS
+ ΔyfhS
YpsA-GFP
ΔyfhS
YpsA
YpsA-GFP
ΔyfhS
ΔyfhS

Cell length (μm)

0
5
10
15
20
25

bioRxiv preprint doi: https://doi.org/10.1101/2020.02.12.946632. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder. All rights reserved. No reuse allowed without permission.
Supplemental Data

Suppressors of YpsA-mediated cell division inhibition in *Bacillus subtilis*

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<table>
<thead>
<tr>
<th>Strain</th>
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<th>Reference</th>
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<td>RB301</td>
<td>any:E:amyE::P_hyperspank-ypsA R111P-gfp spec</td>
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<tr>
<td>RB327</td>
<td>any:E:amyE::P_hyperspank-ypsA G132E-gfp spec</td>
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<td>RB328</td>
<td>any:E:amyE::P_hyperspank-ypsA R111P-gfp spec</td>
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<tr>
<td>RB334</td>
<td>yfhS::erm</td>
<td>Derived from BKE08640 (BGSC*)</td>
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<td>RB288</td>
<td>yfhS::erm; amyE::P_hyperspank-ypsA spec</td>
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<td>RB289</td>
<td>yfhS::erm; amyE::P_hyperspank-ypsA-gfp spec</td>
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<td>RB409</td>
<td>yfhS::erm; bkdB::Tn917::amyE::P_hyperspank-ypsA spec</td>
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<td>RB410</td>
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<td>RB411</td>
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<td>RB412</td>
<td>yfhS::erm; amyE::P_hyperspank-ypsA-gfp flag spec</td>
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<tr>
<td>RB413</td>
<td>yfhS::erm; amyE::P_hyperspank-ypsA-gfp-flagspec</td>
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<td>RBSS6E11</td>
<td>amyE::P_hyperspank-ypsA-gfp** spec</td>
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* Bacillus Genetic Stock Center
** Strain carries suppressor mutation

Oligonucleotides used in this study:

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<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
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<td>oR24</td>
<td>GCCGCGATGCCTTATTTGTTAGAATGTCATCCGATGCC</td>
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<td>oR106</td>
<td>AAATAGCGACGATCGGAGGCACGACACCTCTAGAAGTAT</td>
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<td>oR212</td>
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<tr>
<td>oR680</td>
<td>AATAAAGCTAGCTTATACGTAAGAGAAGCCGCGTGCGTGCTCT</td>
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</table>
Figure S1

A

- Inducer  + Inducer

YpsA-FLAG

YpsA-GFP-FLAG

B

- Inducer  + Inducer

WT

YpsA

YpsA-GFP

YpsA-FLAG

YpsA-GFP-FLAG

C

YpsA-FLAG

YpsA-FLAG

yfhS + YpsA-FLAG

yfhS + YpsA-GFP-FLAG

α-FLAG

α-SigA

FLAG/SigA ratio

0.80  1.59  0.88  0.56
Figure S2

Plate on solid medium containing IPTG

Isolate chromosomal DNA from colonies that form

Transform into fresh PY79, screen for amyE integration

Screen resulting transformants for lethality on solid medium with IPTG

If lethal, classify as extragenic and return to original strain

If able to grow, classify as intragenic

Screen resulting strain amyE integration and for growth on solid medium with IPTG

If able to grow, classify as extragenic

If lethal, classify as intragenic

Isolate chromosomal DNA from colonies that form

Whole genome sequencing

Swap $P_{IPTG}$-ypsA-gfp spec with catR

Transform strain with $P_{IPTG}$-ypsA-gfp spec

amyE::P

amyE::catR

amyE::P

amyE::catR

Plate on solid medium containing IPTG

Isolate chromosomal DNA from colonies that form

Transform into fresh PY79, screen for amyE integration

Screen resulting transformants for lethality on solid medium with IPTG

If lethal, classify as extragenic and return to original strain

If able to grow, classify as intragenic

Screen resulting strain amyE integration and for growth on solid medium with IPTG

If able to grow, classify as extragenic

If lethal, classify as intragenic

Isolate chromosomal DNA from colonies that form

Whole genome sequencing

Figure S2
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