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Role of Amphipathic α-helix and Division-related Proteins in *Bacillus Subtilis*

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Abstract. In many bacteria, cell division is initiated by a tubulin-like protein FtsZ, which forms a ring structure known as the Z-ring at midcell. FtsA and SepF are important membrane anchor of Z-ring, which exists widely in bacteria species and binds to the membrane by a C-terminal amphipathic α-helix in a membrane potential-dependent manner. It reported that amphipathic α-helix binds to the leading edge of developing septum with an intrinsic preference. These discoveries led to a hypothesis, proteins of amphipathic helix might not only function as membrane anchor of FtsZ, but also participating the regulation of septum synthesis. Several variants were made by genetic techniques, and cell length measurements were performed by fluorescence microscopy and ImageJ. All helix variants were found viable, the amphipathic α-helix does affect the cell division but does not affect the functionality.

Keywords: *Bacillus Subtilis,* microbial proteins

1 Introduction

Bacillus subtilis is a Gram-positive and catalase-positive bacterium, found in soil and the gastrointestinal tract of ruminants and humans. *B. subtilis* has been classified as an obligate aerobe in the past, though evidences prove that it is a facultative aerobe [1]. As a member of Bacillus genus, *B. subtilis* is rod shaped, and can form a tough, protective endospore in order to tolerate extreme environmental conditions [2]. It has been deemed to be the best model organism to study chromosome replication and cell differentiation in Gram-positive bacteria. Besides, it has been proven highly amenable to genetic manipulation, and has become extensively adopted as a model organism for laboratory studies.

B. subtilis can divide into two symmetrical daughter cells via binary fission, or divide asymmetrically by producing a single endospore that can remain viable for decades and is resistant to harsh environmental conditions such as drought, salinity, extreme pH, radiation, and solvents [3]. Replication of *B. subtilis* single circular chromosome also initiates at a single locus, the origin (oriC) [4]. Replication proceeds bidirectionally and two replication forks are ongoing in clockwise and counterclockwise directions along the chromosome. The DNA replication is about 45 minutes, and preceded and followed by periods without DNA synthesis of about 10 minutes [5]. Besides, DNA segregation is finished shortly after termination of DNA replication indicating that replication and segregation occur simultaneously.

After chromosome segregation, the cell division in *B. subtilis* is conducted by the divisome, which is a dynamic protein hyperstructure facilitating the constriction during cell division. Most of the genes involved in divisome formation were originally identified as temperature sensitive mutations, as cells continue to grow filamentous and division is hampered at non-permissive temperatures. Therefore most genes involved are named as Fts, which means filamentous temperature sensitive [6].

The first protein known is FtsZ, which forms the Z ring by GTP-dependent polymerization on the inner side of the cytoplasmic membrane and subsequently constricts during cytokinesis. As the initial and critical event in cell division, the formation of Z-ring is strictly regulated. Positive regulating proteins, such as FtsA and ZapA, promote FtsZ ring formation and stabilize the ring during assembly of the divisome. Negative regulating proteins, such as EzrA, MinC, are responsible for preventing FtsZ assembly at inappropriate locations [7]. Besides, proteins such as FtsL, DivlB, DivlC, FtsW, PBP2B also play important roles in cell division.

FtsA is essential in *B. subtilis*, and interacts directly with FtsZ before Z-ring formation [8]. The structural features of FtsA have been determined from Thermotoga maritima, indicating FtsA is an

ATP-binding protein belonging to the actin family [9] [10]. By using electron microscopy, it has been observed that FtsA forms filaments inside the cells, and these FtsA filaments may form an "A-ring" which attaches Z-ring to the membrane [10]. *B. subtilis* FtsA was suggested to self-assemble in a concentration dependent manner into polymeric structures consisting of filaments and bundles [11]. Interestingly, several evidences suggest that FtsA binds to the membrane with an amphipathic helix located at the C-terminus and tethers FtsZ to the membrane by interacting with its C-terminal tail [11] [12].

SepF is also a membrane anchor of FtsZ. SepF is highly conserved in Gram-positive bacteria and cyanobacteria, and it has an apparent functional overlap with FtsA [13]. SepF was found to display FtsA activities: FtsZ binding, polymerization and membrane binding [10]. This might be the reason why bacterial species that do not contain FtsA, such as the Mycobacteria and Streptomycetes, do have SepF homologs. Moreover, SepF is important for the synthesis of smooth and regular septa [13] [14]. The absence of SepF result in slightly elongated cells, but unusually thick and badly malformed septa [15]. Biochemical analysis revealed that the membrane-binding domain of SepF is an amphipathic α-helix at the N-terminus [14].

Both FtsA and SepF process an amphipathic helix for membrane binding. These amphipathic helices bind stronger to convex membrane surfaces due to space created between lipid molecules by curvature [16]. It seems that both FtsA and SepF bind to the leading edge of nascent septum with an intrinsic preference, since the amphipathic helixes prefer positively curved membrane due to relaxed lipid density. Therefore, the membrane anchors FtsA and SepF might play a role in keeping the Z ring positioned at the edge of the developing septum during cell division by this intrinsic preference.

2 Material and Methods

2.1 Bacterial Strains and Antibiotics

Bacterial strains and supplements used in this project are listed in Table 1. All strains were grown in LB at 37°C with continuous shaking. Appropriate supplements were added when required, such as antibiotics, IPTG, glucose. The glycerol stocks of strains (final glycerol concentration 20%) were made for storage. Aliquots were frozen in liquid nitrogen and stored at -80°C culture mediums.

Table 1. Strains and supplements in this project

Supplements	Stock solution	Working concentration
Ampicillin	50 mg/ml	100 μg/ml
Chloramphenicol	10 mg/ml	5 μg/ml
Spectinomycin	100 mg/ml	100 μg/ml
Kanamycin	25 mg/ml	5 μg/ml
Erythromycin	20 mg/ml	0.5 μg/ml
Glucose	40% glucose	0.5%
IPTG	1M	0.1 mM

2.2 Genetic Methods

2.2.1 Polymerase chain reaction (PCR)

PCR was performed by 0.8 μl dNTPs, 1 μl primers, 1 μl template and New England Biolabs HF or GC buffer. The final volume is 20 μl. In this project, PCR was used for cloning SepF and FtsA fragments, and colony PCR was used for checking plasmid integration in this project.

Table 2. The setting of PCR amplification

PCR mixture		PCR setting	
Nuclear-free water	11.7 μl	Fragment	vector

Buffer HF	4 µl	98 °C	5 min	5 min
dNTPs	0.8 µl	Cycle numbers	35 min	35 min
Primer for	1 µl	98 °C	1 min	1 min
Primer rev	1 µl	50 °C	1 min	1 min
Template	1 µl	72 °C	2 min	5 min
MgCl2	0.5 µl	72 °C	10 min	10 min
Polymerase	0.2 µl	4 °C	∞	∞

The PCR products were checked by agarose gel electrophoresis. The fragments were mixed with DNA loading buffer in 1% agarose gel, which was made with 1x TAE buffer and GelRed nucleic acid dye. The gels were run in 1x TAE buffer at 80V. The DNA bands were visualized with UV transilluminator. The PCR products were purified by Wizard SV gel and PCR Clean-Up System and stored in -20 °C.

Gibson assembly was performed by mixing plasmid backbone pMUTIN4 and variants SepF fragments with 15 µl Gibson assembly reagent (20% PEG-8000, 600 mM Tris-HCl pH 7.5, 40 mM MgCl2, 40mM DTT, 800mM each of the 4 dNTPs, 4 mM NAD, 4 U µl–1 ExoIII, 4 µl of 40 U µl–1 Taq DNA ligase and 0.25 ml of 5 U µl–1 Ab-Taq polymerase) [17], and then incubated the mixture in PCR for 45 minutes at 50 °C. The 3µl Gibson products were added into 100 µl competent Top10 cells. The DNA integration was achieved by heat shock, and then plate the mixture in selective plates at 37°C. The colonies were checked by colony PCR and sequenced. Plasmids which showed positive results in colony PCR were isolated and transformed into storage.

2.2.2 Isolation of chromosomal DNA

Chromosome DNA of *ΔftsA* and *ΔsepF* were isolated from 10 ml overnight culture. The protocol of DNA isolation is attached as appendix 1.

2.2.3 Transformation

TYM broth and minimal medium were used for making calcium competent *B. subtilis* cells. The protocol is attached in appendix 2. The DNA to be transformed was added to competent cells and incubated in for 45 minutes at 37°C under continuous shaking, followed by plating 100 µl fractions on selective plates.

2.3 Protein Purification

Chimera SepF proteins were purified by AKTA protein purification system. *B. subtilis* TOP10 strian cultured overnight in 20 ml LB with 100 µl/ml ampicillin at 37 °C under continuous shaking. Then the overnight culture was diluted 1:100 and grown until OD600 = 0.4 in 4L fresh LB containing ampicillin for 3 hours. Expression of maltose-binding protein (MBP)-tagged SepF proteins was induced by addition of 0.1 mM IPTG for 1 hour. Cells were harvested by centrifugation and 1× washing with PBS+PMSF. Frozen the pellet in liquid nitrogen for further use.

Cell pellets were dissolved in 20 ml buffer AF (50 mM Tris-HCL, pH 7.4, 200 mM KCL, 5 mM EDTA, 0.5 mM DTT) with one mini protease tablet and disrupted by French press (4 times in 800 psi). Cell debris was removed by ultracentrifugation at 21000 rpm 4 °C for 1h. The supernatant was filtered through 0.2 µl filter and loaded onto a Tricorn 10/20 column (GE Healthcare) packed with 2 ml amylose resin (New England Biolabs) equilibrated with buffer AF. The column was washed with 5 column volumes buffer AF, followed by buffer BF (50 mM Tris-HCl, pH 7.4) until the baseline was stable. MBP-tagged SepF variants were eluted with buffer BF containing 10 mM maltose. MBP-tagged SepF was digested with factor Xa protease (New England Biolabs) in the presence of 2 mM CaCl2 at 4°C overnight.

Proteins were separated from MBP and factor Xa by centrifugation at 10,000 rpm. Protein pellets were rinsed and dissolved in buffer BF and stored as 20 µl aliquots at -80 °C until further use.

2.4 Microscopy

Samples were mounted on microscope slides coated with a thin layer of 1% agarose. The cell membrane was stained with mitotracker green. Images were acquired with Nikon Eclipse Ti fluorescence light microscope. The cell length was measured with ImageJ.

To prepare protein sample for transmission electron microscopy, the protein drops were mounted on glow discharged and carbon coated grids for 1 minute in room temperature, excess liquid was removed with paper tissue. Then the grids were negatively stained with 100 µl 2% uranyl acetate drop by drop and excess staining solution was removed with paper tissue. Grids were air-dried and examined with JEOL 1010 at an electron voltage of 80 kV.

3 Results

3.1 Growth Curve

Expression of the SepF amphipathic helix variants was induced with IPTG. In order to find the optimal IPTG concentration for further experiments, growth experiments for Strains (wildtype, ftsA::cam, sepF::spc) were performed at both sterile 96 well plate and LB agar plates, and each culture was inoculated with different concentration of IPTG and incubated in 37°C overnight.

As the result, the optimal IPTG concentration of 96 well culture for *ΔftsA* strain is 0.1 mM and for *ΔsepF* strain is 0.05 mM. However, WT-like growth was observed at lowest concentration of IPTG at plate reader. The growth experiments on agar shows good condition in both 0.05 mM and 0.1mM concentrations, therefore 0.1mM has been selected as best concentration for all strains.

3.2 Cell Length of Helix Variants in Liquid LB Medium

To figure out the weather amphipathic helix homologues affect Z-ring placement and septum synthesis, we measured cell length of variants. Strains in Table 3 were grown in LB with their optimal IPTG concentration. The cells were incubated in 37°C overnight with continuous shaking, then 1:10 diluted the overnight culture with fresh LB medium. To make sure all cells were imaged in healthy condition, the cultures were grown until log phase, when the OD600 was around 0.4.

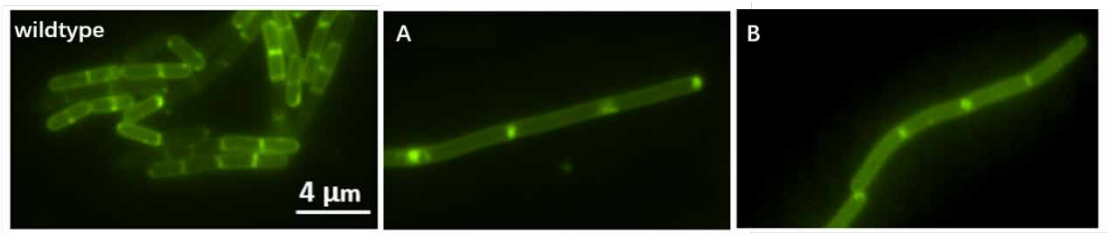
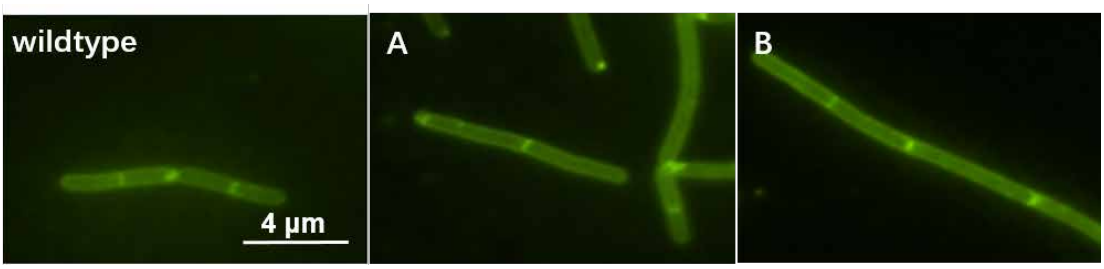


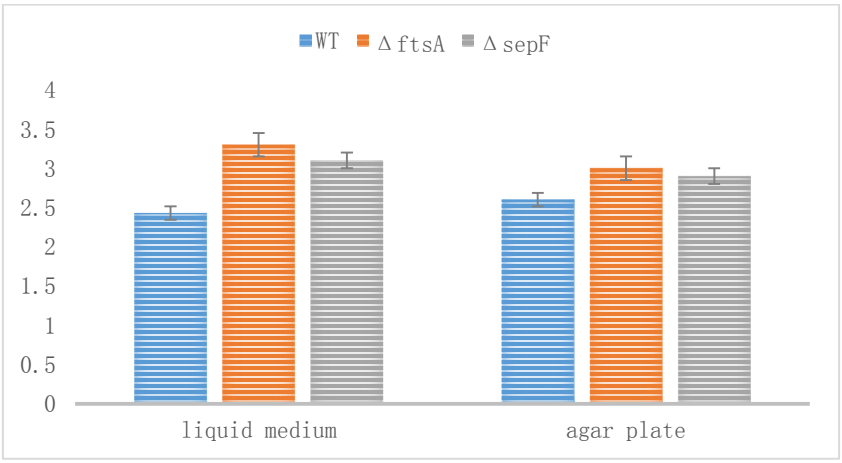
Figure 1. Florescent microscopy images and cell length data for different helix variants in liquid LB with IPTG. The cells were stained by mitotracker green dye and taken pictures by Nikon Eclipse Ti microscopy. *ΔftsA* strain (A) and *ΔsepF* strain (B) were observed longer cell length than wildtype strain.

The wildtype showed similar length with *ΔsepF* strain, which means the mutation of SepF did not make big change for the length of cell. However, longer cell length was observed in *ΔftsA* strain indicates that the knockout of ftsA leads to morphology deformation. To determine the influence of amphipathic helix variants on cell length, strain with double deletion of sepF and ftsA was attempted to make but fail. Thus the deficiency of both FtsA and SepF is lethal, and their amphipathic helix does affect cell division by the strength of binding.

Interestingly, different morphology of mutant strain was observed on LB agar plate after *ΔftsA* transformation. The cells were elongated and curved which has not been reported before. Thus, we compared the cell length of same strains in both liquid and solid medium. After inoculated the colony to fresh liquid LB, the unusual morphology of *ΔftsA* cells was less pronounced. The reason for this remains to be determined in future studies. Then the same cell length measurement experiments were tested on strains grown in LB agar plate too.



(1)



(2)

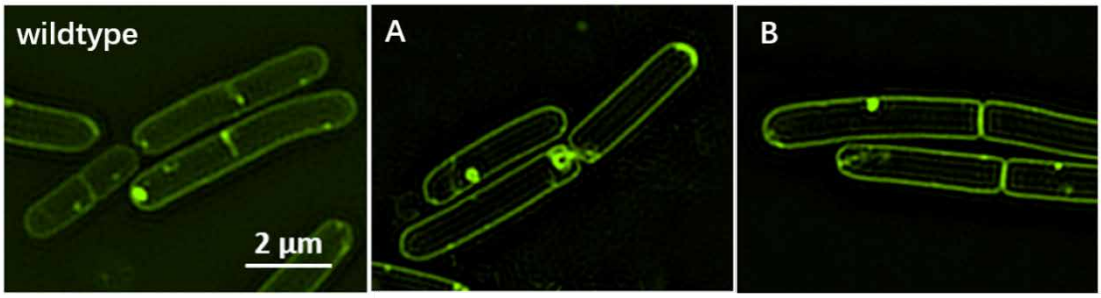
Figure 2. Florescent microscopy images of agar plate culture and the comparison of cell length between two culture conditions. (1) The cells were stained by mitotracker green dye and imaged by Nikon Eclipse Ti. ImageJ was utilized for cell length measurement. (2) Compare the difference between liquid and solid cultures in cell length by average cell length, the results between liquid and plate cultures were quite consistent. But the plate-cultured strains were mildly identical to each other and less differences observed in cell length.

The cell length of bacteria grown in liquid or solid medium shows identical trend in cell morphology. The knockout of membrane anchor triggered to longer cell, and mutant control so was too. Remarkably, cells grown on plate illustrated lower variance in different strains, approximately 35% lower than liquid cultures. This indicating the fluid culture condition gave bacteria more variable possibility in environment.

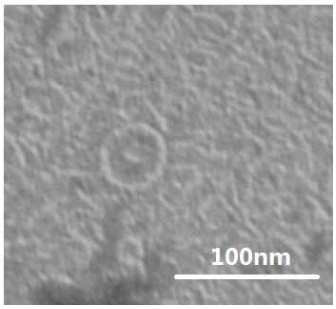
3.3 SepF Ring Diameter Measurement

Purified SepF forms ring structure in vitro, and it was suggested that there is a correlation between SepF inner ring diameter and septum thickness. SepF is suspected to be responsible for regulating the septum diameter in Gram-positive bacteria. The *B. subtilis* SepF was isolated and purified from TOP10 wildtype, SIM and TEM was performed to measure the septum size and diameter of SepF ring structure.

100 septum of each strain and 10 rings were measured by ImageJ. The average septum of variants was approximately 40nm, and the average ring diameter of SepF was 38nm. The highly consistent result of SepF size and septum thickness indicating the amphipathic helix consisted by FtsA and SepF is responsible for regulating septum width. The helix structure would not only function as membrane anchor of Z-ring, but also participating the regulation of septum synthesis and ensuring an appropriate septum diameter.



(1)



(2)

Figure 3. The bacterial septum and SepF protein ring structure by microscopy. (1): SIM images of strains cultured above. Septum in different variants were observed in identical sizes. (2): Protein samples were negatively stained with 100 μl 2% uranyl and examined by JEOL 1010 at an electron voltage of 80 kV.

4 Discussion

Cell division is conducted by the divisome, which is a dynamic protein hyperstructure facilitating the constriction during cell division. The protein machinery of divisome was well studied in the Gram-positive *B. subtilis*. In many bacteria, cell division is initiated by a tubulin-like protein FtsZ, which forms a ring structure known as the Z-ring at midcell. The *B. subtilis* proteins recruited to divisome in early stage, such as FtsA, SepF, EzrA, assisting the organization of Z-ring and anchor it to the cytoplasmic membrane. And late stage proteins like PBP1, PBP2B, DivIB and DivIC, controlling constriction and septum formation [18].

The membrane anchors FtsA exists widely in bacteria species and binds to the membrane by a C-terminal amphipathic α-helix in a membrane potential-dependent manner [19]. Another essential membrane anchor of *B. subtilis* is SepF, which has overlap function with FtsA. Actually, bacteria lacking FtsA always contain SepF homologs. In fact, double deletions of ftsA and sepF is lethal, indicating that SepF is the only protein that can serve as single membrane anchor of the Z-ring in *B. subtilis*. The SepF forms ring structures in vitro, and it contains membrane binding domain and Z-ring binding domain by N-terminal amphipathic α-helix. Based on our result, the amphipathic helix anchoring FtsZ to membrane affects cell length, but does not influence functionality. The helix structure would not only play a role in mediating the interaction of protein and membrane, but also regulating the septum morphology and adjusting septum diameter.

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Appendix 1. Chromosomal DNA isolation (070620)

1. Spin down 2 ml B. subtilis culture grown overnight at 30°C in PAB (contains sugar which suppresses sporulation).
2. Wash 1 x in 1 ml TES buffer
3. Resuspend pellet in 750 µl TES, add 25 µl Lysozyme solution and 25 µl RNase solution, and incubate 30 min at 37 °C.
4. Add 25 µl Pronase, mix (all mixing is done by shaking) and add 30 µl Sarkosyl (30 %), and mix, continue incubation at 37 °C for 60 min
5. Add 250 µl phenol, mix and add 250 µl chloroform. Centrifuge 4 min (not shorter! the cleaner the DNA, the better it dissolves) after 2 min mixing.
6. Take up upper layer with decapitated blue tip and mix with 500 µl chloroform in 2 mL Epp. After 2 min centrifuge 4 min
7. Take of the up upper layer and add to 2 ml epp tube. Fill up with isopropanol. Mix until a clear white DNA cloud appears
8. Spin short (1 sec) and remove white DNA pellet with pipette and add to 1.5 ml epp tube containing 70 % ethanol.

9. Wash 2 x with 70 % ethanol (pellet sinks, let salt diffuse for 1 min).
10. Remove as much ethanol solution after last wash step (short spin).
11. Solubilize in 0.1 ml mQ

- Check DNA for absence of spores by plating 20 µl on selective plates. Alternatively, solubilise in 400 TES and filter sterilize.
- TESbuffer: 10 mM Tris-HCl pH 8, 1 mM EDTA, 100 mM NaCl (for 100 ml: mix 1ml 1 M Tris-HCl pH 8 + 200 µl 0.5 M EDTA + 2 ml 5 M NaCl add mQ to 100 mL)

Appendix 2. Transformation of competent B. subtilis cells

MM competence medium:

10 ml	SMM medium
0.125 ml	sol E = 40 % glucose
0.1 ml	tryptophane solution
0.06 ml	sol F = 1 M MgSO4
0.01 ml	20 % CAS
0.005 ml	0.22 % Fe-NH4-citrate

Starvation medium:

10 ml	SMM medium
0.125 ml	sol E = 40 % glucose
0.06 ml	sol F = 1 M MgSO4

- inocculate 10 ml overnight culture in MM competence medium.
- take ~1 ml overnight culture and inocculate 10 ml MM competence medium.
- grow 3 hours.
- add 10 ml prewarmed starvation medium, and continue for 1.5 hours (cells can also be competent after 0.5-1 hr. If transformation is important, try different starvation times).
- mix 10 microliter DNA with 0.4 ml competent culture, and shake for 45 min to 1 hour, before plating on selective medium.

10x SMM	g/l
(NH4)2SO4	20
K2HPO4 / K2HPO4 3H2O	139 / 193
KH2PO4	60
NaCitrate 2H2O	20

Sol F:	
1M MgSO4 7H2O	246.5 g/l

Tryptofaan	5 mg/ml

