FINDING THE MISSING LINK BETWEEN CELL DIVISION AND SPORULATION IN GRAM-NEGATIVE BACTERIA

A Thesis

by

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ABSTRACT

Many bacteria survive harsh environments and facilitate their dispersion by developing vegetative cells into dormant spores. Gram-positive bacteria, such as *Bacillus subtilis*, have been used as model organisms for bacterial sporulation. In these bacteria, vegetative cells initiate sporulation by DNA replication followed by asymmetric cell division, after which the smaller daughter cells mature into spores. Some Gram-negative bacteria, such as *Myxococcus xanthus*, can also form spores. In contrast to Gram-positive bacteria, DNA replication, but not cell division, is required for *M. xanthus* sporulation. Instead, the entire rod-shaped vegetative cells directly transform into spherical spores. I hypothesize that the sporulation of Gram-positive and negative bacteria has evolved from a common mechanism that involves cell division. Amidases are the cell wall hydrolases which could potentially participate both the separation of daughter cells during division and the rod-to-sphere transition during sporulation. To search for this missing link between sporulation and division in *M. xanthus*, I systematically knocked out all the amidases in *M. xanthus* and identified one dual-functional amidase, DfaA, essential for both division and sporulation. I propose to investigate the spatial and temporal regulation of DfaA and elucidate the hidden connection between cell division and sporulation in Gram-negative bacteria.

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I'd like to humbly profess my gratitude to God who has never left my sight and till this day has been my main source of strength. My mission in this world will always be guided by him and he has opened the doors every single step of the way.

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Contributors

With the guidance and support from Dr. Beiyan Nan, all the work conducted for the thesis was completed independently by me.

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NOMENCLATURE

Peptidoglycan cell wall (PG)

Peptidoglycan synthases (PG synthases)

Peptidoglycan hydrolases (PG Hydrolases)

Hydrolases -N-acetylglucosaminidase (Tpases)

N-acetylglucosamine (NAG)

N-acetyl muramic acid (NAM)

TABLE OF CONTENTS

Page

ABSTRACT…... ii

ACKNOWLEDGMENTS.. iii

CONTRIBUTORS AND FUNDING SOURCES………………………....................... iv

NOMENCLATURE………………………………………………………................. v

TABLE OF CONTENTS ………………………………………………….................. vi

LIST OF FIGURES ……………………………………………………….................. vii

INTRODUCTION ………………………………………………………….................. 1

MATERIAL AND METHODS……………………………………………..................... 4

2.1 Sporulation assay done for all putative amidases...4
2.2 Systematic knockout of the genes encoding M. xanthus PG amidases.................5

RESULTS AND DISCUSSION……………………………………...…............... 6

3.1 Part of the cell division system is required to initiate M. xanthus sporulation......6
3.2 DfaA (MXAN_2003) could be the missing link between cell division and sporulation...6

PROPOSED EXPERIMENTS ……………………………………….................. 12

4.1 Confirm the functionality of DfaA-PAmCherry..12
4.2 Investigate the localization of DfaA during division and sporulation..................12
4.3 Elucidate how cell division system initiates sporulation.....................................12

REFERENCES……………………..……………………………………….................. 14

LIST OF FIGURES

Page

Figure 3.1.1. Wild type *M. xanthus* cells complete sporulation within 2 h after glycerol....7

Figure 3.1.2. Part of the cell division function is required for *M. xanthus* sporulation....8

Figure 3.1.3. Cell division is only required for the initiation of *M. xanthus* sporulation.... 9

Figure 3.2.1. DfaA (MXAN_2003), a putative PG amidase, is involved in cell division.... 10

Figure 3.2.2. DfaA is required for glycerol-induced sporulation.... 11

1. INTRODUCTION

To survive harsh environmental conditions, many rod-shaped bacteria can form oval or spherical spores, which are highly developed, dormant cells. Gram-positive bacteria, such as *Bacillus subtilis* and *Clostridiodes difficile*, have been used as the model organisms for bacterial sporulation. In these bacteria, the key step of sporulation is asymmetric cell division. After DNA replication, cells form septa close to one cell pole, which creates a divide between the mother cell compartment and the small forespore. Later only forespores mature into spores (2,5,10). The rod-to-oval transition during the sporulation of Gram-positive bacteria is achieved by cell division. For both *B. subtilis* and *C. dificile*, the PG structures from the vegetative cells become germ cell walls in spores and the mother cells also deposit a thickened layer of PG called cortex to enclose the original PG (16,17).

Many Gram-negative bacterial also form spores. *Myxococcus xanthus* is a rod-shaped Gram-negative bacterium. In laboratory conditions, adding simple chemicals, such as glycerol and DMSO into liquid *M. xanthus* culture can induce quick sporulation. In contrast to Gram-positive bacteria, *M. xanthus* sporulation does not require cell division. Instead, the entire vegetative cells transform into spores. Biochemical and electron microscopy studies revealed that these spores are free of PG (1,9,12,20,21). As the peptidoglycan (PG) cell wall determines cell shape (6,21), the rod-to-sphere transition results from the total degradation of PG. Strikingly, DNA replication is still required for *M. xanthus* sporulation (7,11,18). This observation could suggest that despite to its absence, cell division could still play a role in *M. xanthus* sporulation. If this hypothesis is proved true, it could further suggest that sporulation of both Gram-positive

and negative bacteria could have evolved from a common mechanism. Indeed, there is a hypothesis that sporulation is the onset of the evolution of all bacteria (4,8,16,17).

PG in most bacteria is a rigid layer that protects cells against osmotic pressure and determines cell shape. PG is a net-like polymer of glycan chains of alternation of N-acetylglucosamine (NAG) and N-acetyl muramic acid (NAM) that are crosslinked by peptides (6,11). PG is under the coordinated control of polymerases and hydrolases. PG polymerization during both vegetative growth and cell division (the growth of septum) is carried out by peptidoglycan glycosyltransferase (GTases) that elongate the glycan chains and transpeptidases (TPases) that form peptide crosslinks (3). To allow the insertion of new PG subunits, hydrolases are also required to break bonds within the existing PG network. Bacteria encode diverse PG hydrolases to tailor the length of the glycan strands and the degree of crosslinking between the peptide stems. Such hydrolases include lytic transglycosylases that cleave glycosidic bounds between NAM and NAG, amidases that remove peptides, endopeptidases and carboxypeptidases that cleave peptide crosslinks at different positions (19).

Among the enzymes that degrade PG, amidases are especially required for the separation of daughter cells during cell division (13,14,15,19,). Thus, I hypothesize that amidases could be the missing link between sporulation and cell division in *M. xanthus* and that the divergence in the regulation of amidases could result in distinct sporulation patterns in Gram-positive and negative bacteria.

To test my hypotheses, I have confirmed that part of the division function is required for *M. xanthus* sporulation. Through systematic knock-out, I have identified DfaA, a d̲ual-f̲unctional a̲midase, essential for both division and sporulation. Based on these observations, I propose to

investigate the localization and dynamics of DfaA using fluorescence microscopy and reveal the regulatory mechanism that underlies the division-free sporulation in *M. xanthus*.

2. MATERIAL AND METHODS

2.1 Sporulation assay done for all putative amidases.

M. xanthus cells were grown in CYE medium to $OD_{600} \leq 1.0$-1.5 and then diluted to OD_{600} 0.5. To induce sporulation, glycerol was added to 0.5 M. To quantify the sporulation progress, cells were imaged at different time points after induction using differential interference contrast (DIC) microscopy and their length to width ration was analyzed using a Matlab script (20).

Table 2.1. Putative amidase genes knocked out and primers used in this study

Gene Knocked out	Primers
mxan_3566	5′-cccaagcttCACTGGATGTCCCCGCCCATG-3′ 5′-cgggatccCACCGTCGGCTTGAGCCCCA-3′
mxan_6372	5′-cccaagcttGGCCTCGCCGAAAGCCAGC-3′ 5′-cgggatccCCGACTTTGTCATGATTCAC-3′
mxan_1104	5′-cccaagcttGGTCCGCTGGCGTTCCCTGTT-3′ 5′-cgggatccGTAGAGCAGCAGCTCCGCCAC-3′
mxan_0345	5′-cccaagcttTGATTCCGGGTGGTTGCACCGT-3′ 5′-cgcggatccTCATCGGCGTACTTCGACAGCAG-3′
mxan_6372	5′-ggggtaccAGGGTTGGAAGGTCCCCGGGTGA-3′ 5′-ggaattcGGCACACCGACGATGCGCAGT-3′
mxan_4838	5′-cccaagcttTCGTGAACGCGCTCGAGAG-3′ 5′-cgggatccAATCTCATTCATGTTGGCCT-3′
mxan_3886	5′-cccaagcttCATCGTGCTGCCCGTGCTGCTG-3′ 5′-cgggatccCGGACGCGGACGTCTCCGCCGA-3′
mxan_2003	5′-cccaagcttCCTCGGCCTCCTGGCCCTGCT-3′ 5′-cgggatccAGGCGCGAGGAATCCGCGTGC-3′
mxan_2814	5′-cccaagcttCTCCGGCAAGGTGGTGTACCT-3′ 5′-cgggatccGAAGCGCACCGCGTCCAGCGACA-3′
mxan_3999	5′-cccaagcttTCTGCTGCTGGGACTGGGACT-3′ LR 5′-cgggatccGCGGACTCGGACCAGAGCAC-3′
mxan_2852	5′-cccaagcttTGACGCAGTGGGATTGGCGG-3′ 5′-cgggatccAGCATCGCCGCGCTGTCGCG-3′
mxan_0987	Left flank: 5′-cccaagcttCAGCACGAAGTCCGTGGTGGA-3′ 5′-cgggatccGAGGAGGGCTTCCAGGGGGTAGA-3′ Right flank: 5′-ggggtaccTGACAGTCGTCTTCCCCTGC-3′ 5′-ggaattcAAGGGACAGCGCGGCATCGG-3′

2.2 Systematic knockout of the genes encoding *M. xanthus* PG amidases.

Twelve genes that are predicted to encode PG amidase in the *M. xanthus* genome were individually knocked out using the plasmid insertion method. The genes knocked out and the primers used are listed in Table 2.1.

3. RESULTS AND DISCUSSION

3.1. Part of the cell division system is required to initiate *M. xanthus* sporulation.

Is there any connection between cell division and sporulation in *M. xanthus*, the Gram-negative bacterium? To answer this question, I investigated the effects of cephalexin, an antibiotic that specifically inhibits cell division (11,12), on *M. xanthus* sporulation. As shown in Fig. 3.1.1, wild type *M. xanthus* cells complete sporulation within 2 h after glycerol induction. However, when cephalexin (80 µg/mL) was added 2 h prior to glycerol induction, cells failed to form spores (Fig. 3.1.2). This result indicates that at least part of the division system is required for the initiation of sporulation. In contrast, when cephalexin was added 1 h after glycerol induction, cells still completed sporulation within 2 h (Fig. 3.1.3). This result indicates that the cell division system is not required after the commencement of sporulation, which is consistent with the observation that *M. xanthus* cells do not divide during sporulation.

3.2 DfaA (MXAN_2003) could be the missing link between cell division and sporulation.

As amidases are required for both the separation of daughter cells during cell division and the degradation of PG during sporulation, I hypothesize that certain amidase(s) could be required for both functions in *M. xanthus*. To identify such amidase(s), I systematically knocked out the Twelve genes that are predicted to encode PG amidases. For the resulted mutants, I analyzed both the morphology of their vegetative cells and their sporulation progress after glycerol induction using DIC microscopy. Among these mutants, only the strain that carries the knockout of *mxan_2003* showed defects in both cell division and sporulation. As the protein product of this gene is a putative <u>d</u>ual <u>f</u>unctional <u>a</u>midase, I propose to name this gene as *dfaA*. As shown in Fig. 3.2.1, vegetative cells of the *dfaA* knock out strain are significantly longer than wild type

cells, indicating that this strain cannot divide normally. Importantly, the *dfaA* knock out cells failed to form spores after glycerol induction (Fig. 3.3.2).

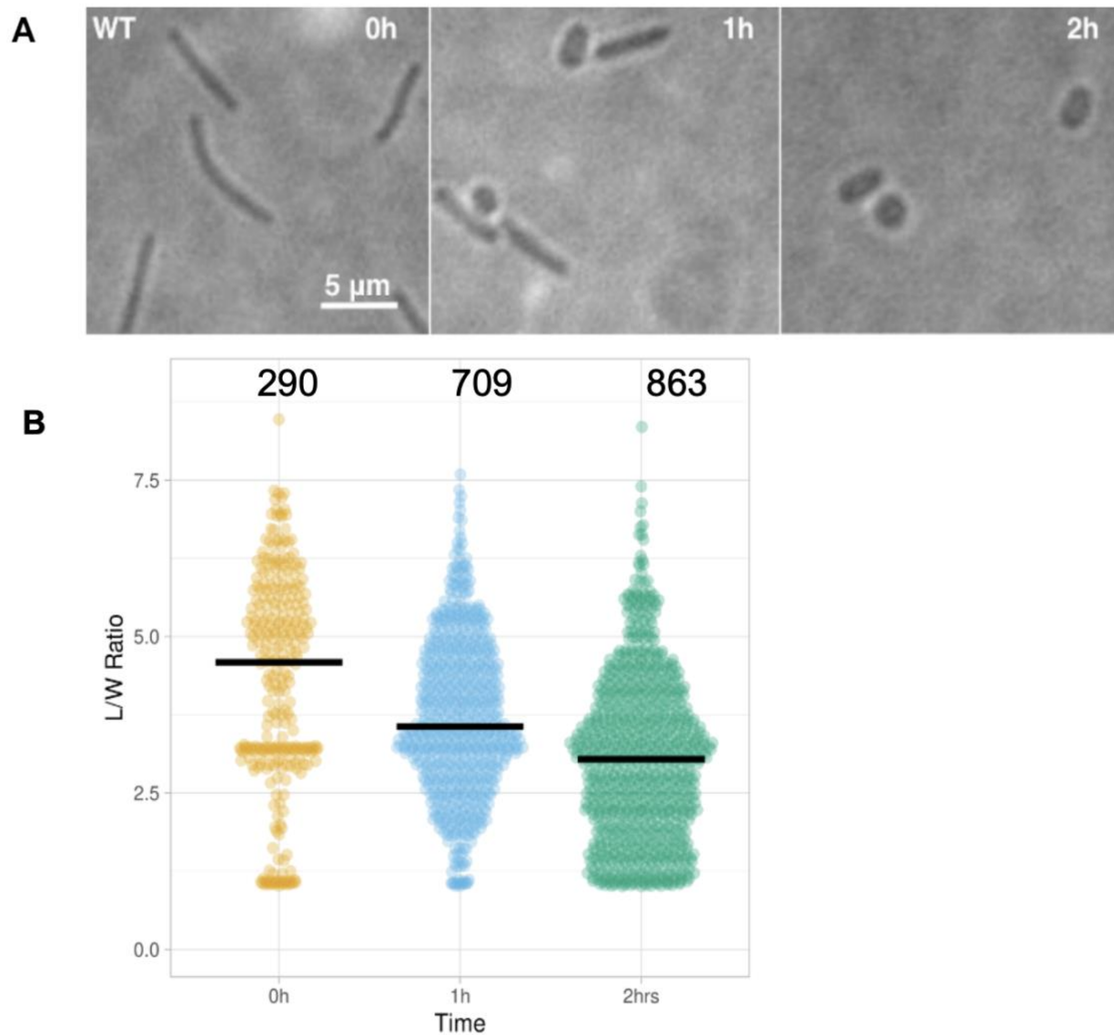


Fig. 3.1.1. Wild type *M. xanthus* cells complete sporulation within 2 h after glycerol induction. (A) Bright field images of cells before (0 h) and after (1 h, 2 h) glycerol induction. (B) Quantitative analysis of the sporulation process using the length/width (L/W) ratio of cells.

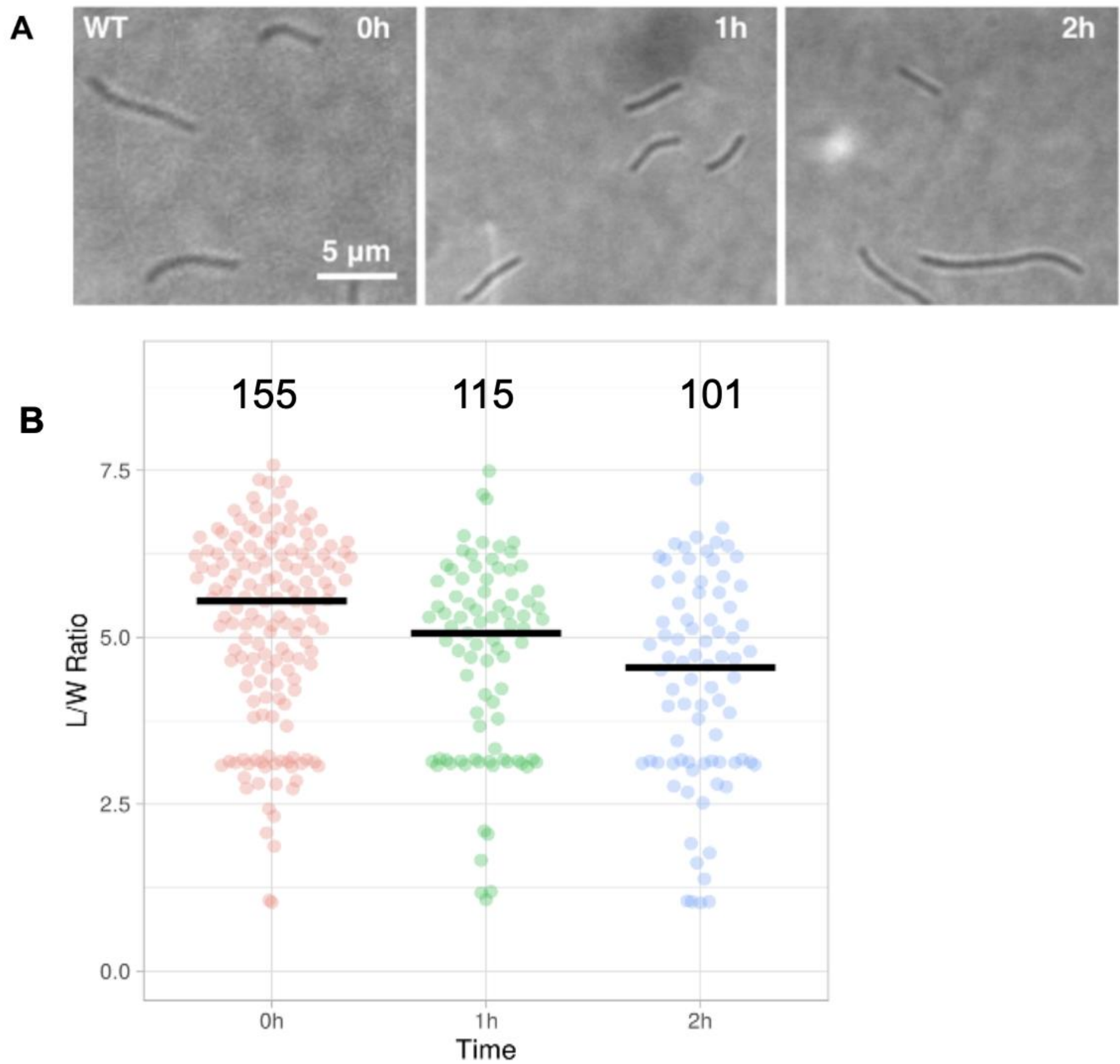


Fig. 3.1.2. Part of the cell division function is required for *M. xanthus* sporulation. When cephalexin (80 µg/mL), an inhibitor of cell division, was added 2 h prior to glycerol induction, cells failed to form spores. (A) Bright field images of cells before (0 h) and after (1 h, 2 h) glycerol induction. (B) Quantitative analysis of the sporulation process using the length/width (L/W) ratio of cells.

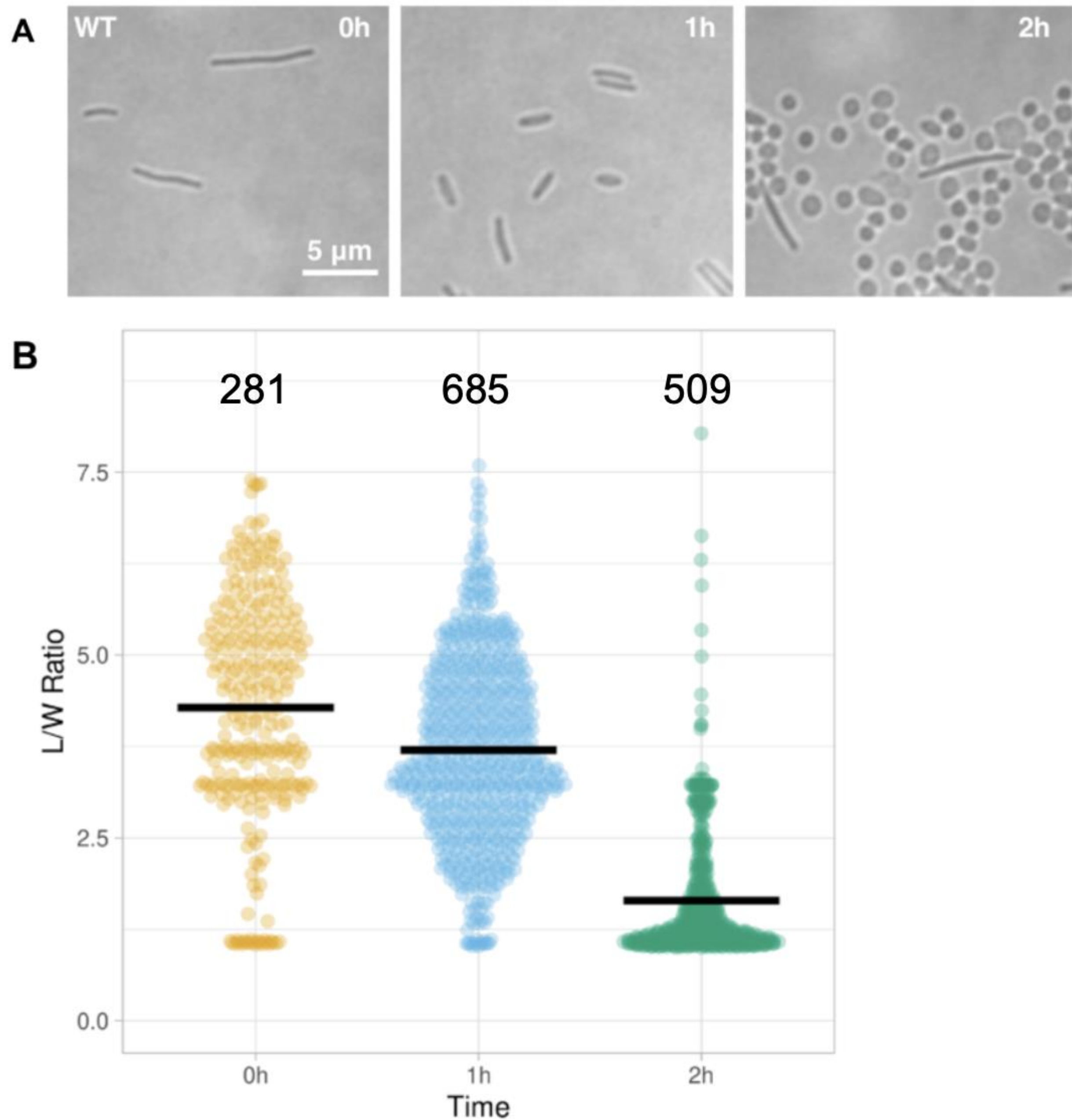
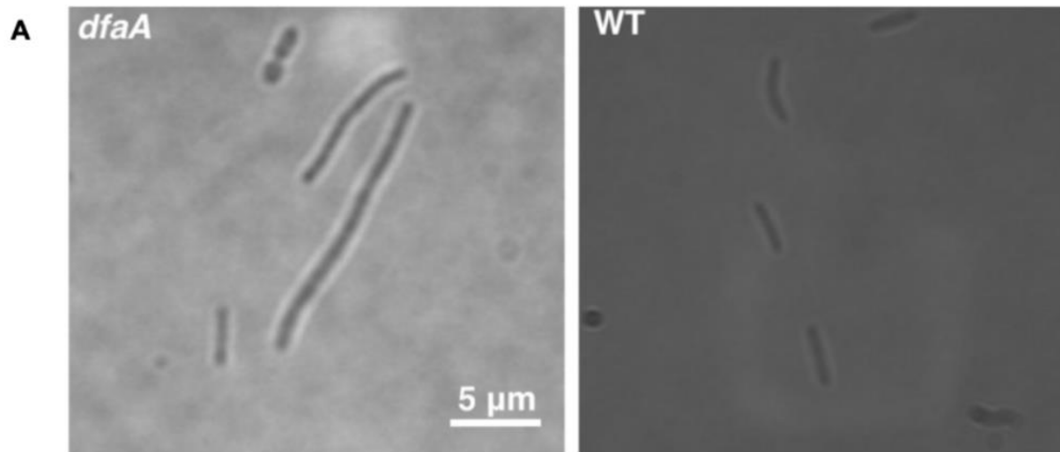


Fig. 3.1.3. Cell division is only required for the initiation of *M. xanthus* sporulation. When cephalexin (80 µg/mL) was added 1 h after glycerol induction, cells formed spores. (A) Bright field images of cells before (0 h) and after (1 h, 2 h) glycerol induction. (B) Quantitative analysis of the sporulation process using the length/width (L/W) ratio of cells.



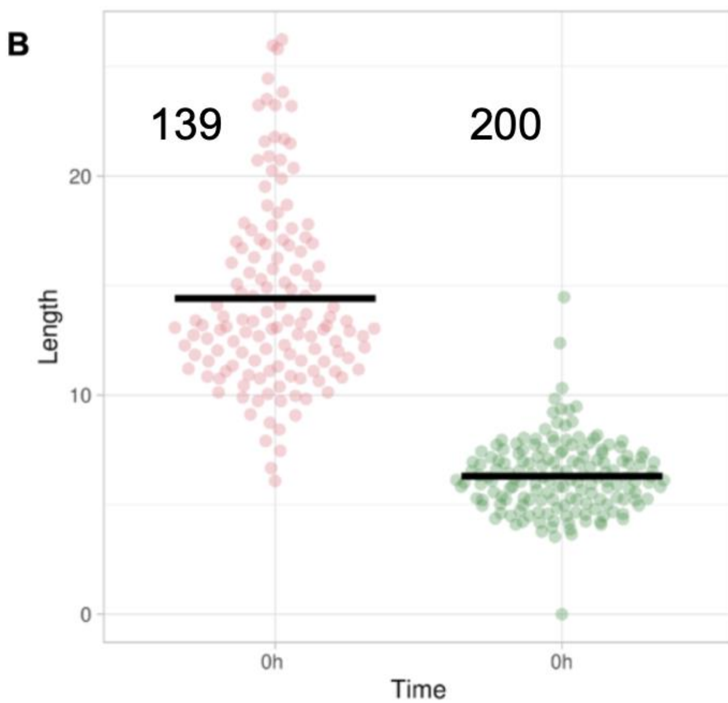
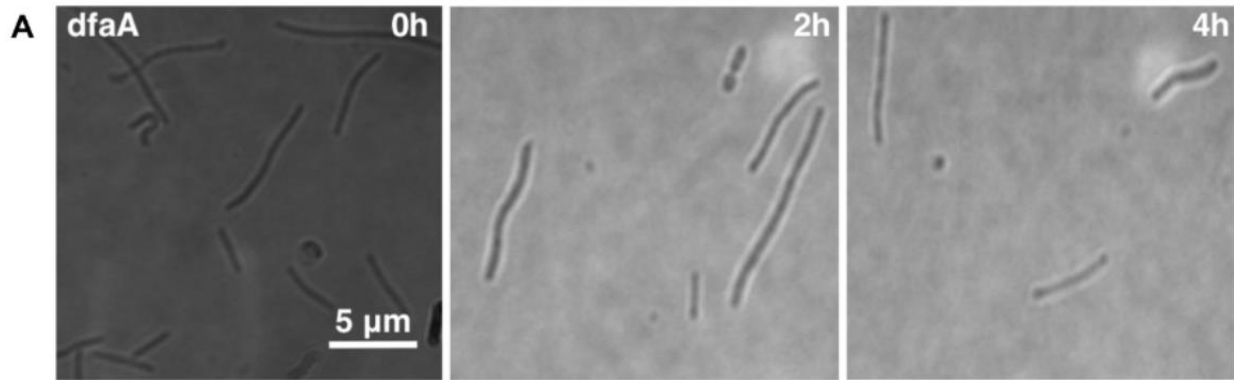


Figure 3.2.1. DfaA (MXAN_2003), a putative PG amidase, is involved in cell division. Compared to the wild type, cells lacking DfaA are elongated. (A) Bright field images of vegetative cells. (B) Quantitative analysis of cell length.



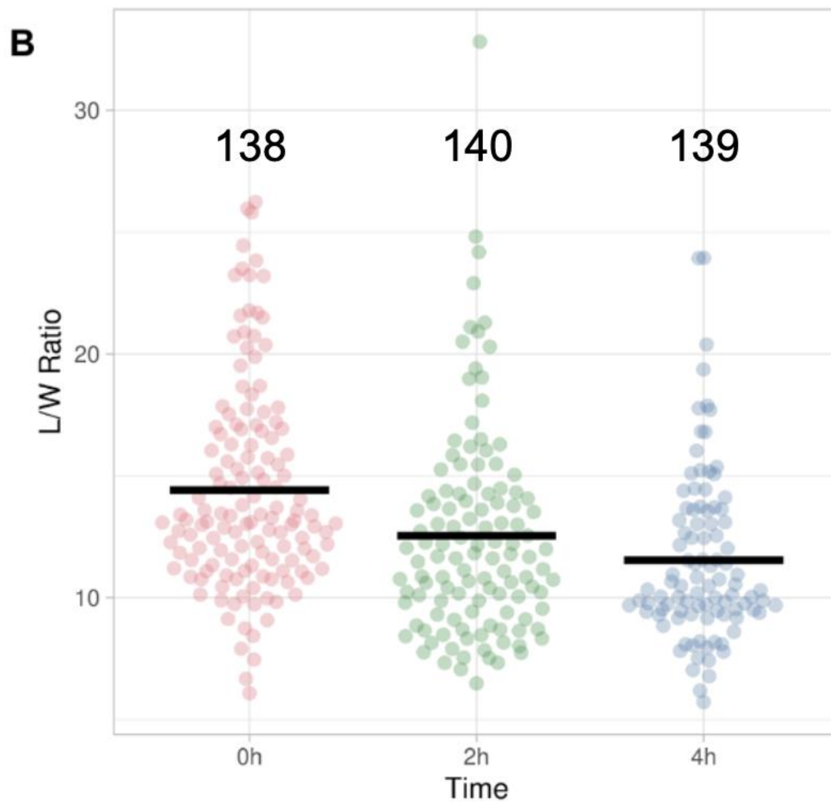


Figure 3.2.2 DfaA is required for glycerol-induced sporulation. (A) Bright field images of cells before (0 h) and after (1 h, 2 h) glycerol induction. (B) Quantitative analysis of the sporulation process using the length/width (L/W) ratio of cells.

4. PROPOSED EXPERIMENTS

To understand how DfaA regulates both cell division and sporulation, I propose to lable DfaA with photoactivatable mCherry (PAmCherry) and express the labled protein as the sole source of DfaA using its native promoter and gene locus. I propose to perform the following experiments:

4.1. Confirm the functionality of DfaA-PAmCherry.

To confirm if the PAmCherry-labled DfaA is functional, I will compare the phenotypes in both division and sporulation between the wild type and the strain expressing DfaA-PAmCherry. DfaA-PAmCherry is functional if the mutant strains divides and sporulates normally. Otherwise, I plan to optimize the labeling strategy, such as moving PAmCherry to the N-terminus of DfaA or change the linker peptide between DfaA and PAmCherry to obtain a fully functional strain.

4.2. Investigate the localization of DfaA during division and sporulation.

Cell division requires highly localized amidase activities at the septum, whereas sporulation requires thorough PG degradation on the entire PG layer. Thus, I hypothesize that DfaA will display distinct localization patterns during cell division and sporulation. To test this hypothesis, I propose to investigate the localization patterns of DfaA-PamCherry in these processes using fluorescence microscopy.

4.3. Elucidate how cell division system initiates sporulation.

To understand how cell division system initiates sporulation, I propose to investigate the effects of cephalexin on the localization and dynamics of DfaA. For instance, does pretreatment with cephalexin inhibits DfaA from distributing to the entire PG layer? After the initiation of sporulation, does DfaA stop responding to cephalexin? I am fully aware that besides localization,

the regulation on DfaA may also occur on molecular dynamics. Compared to the PG structure that is relatively static, the binding of DfaA to PG will reduce the diffusion of individual DfaA molecules. Taking advantage of the PAmCherry tag that can be used for single-molecule tracking, I plan to quantify the diffusion of DfaA molecules using single particle tracking photoactivatable localization microscopy (sptPALM).

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