Mutation Studies of the Gene Encoding YuiC, a Stationary Phase Survival Protein in *Bacillus subtilis*

Doris Quay Huai Xia[1,2*], Amna Qureshi[2], Sanjib Bhakta[2] & Nicholas H. Keep[2]

[1]School of Biosciences and Biotechnology, Faculty of Science and Biotechnology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor Darul Ehsan

[2]Department of Biological Sciences, Birkbeck, University of London, Malet Street, London WC1E 7HX, UK

*Corresponding author email: dorisquay@ukm.edu.my

Abstract

Aims: YuiC is a stationary phase survival (Sps) protein from the Firmicute *Bacillus subtilis* that possesses muralytic activity to cleave bacterial cell-wall peptidoglycan. It has a small lytic transglycosylase (MltA) fold analogous to the resuscitation promoting factors (Rpfs) of Actinobacteria which have a hybrid of a mini lysozyme and soluble lytic transglycosylase (Slt35/70) fold. The present study aimed at identifying key residues of YuiC/Sps that are catalytically active and studying the effect of *B. subtilis* cell growth upon *sps*/*yuiC* deletion.

Methodology & Results: Four forms of mutated *yuiC* were created through Site-directed, Ligase-Independent Mutagenesis Polymerase Chain Reaction (SLIM PCR) that include the substitutions of D129A, D151A, D162A and K102A. These individual mutated *yuiC* genes were cloned and expressed in the *Escherichia coli* BL21 (DE3) expression system and subsequently

purified to homogeneity using affinity, cation exchange and size exclusion chromatography. The D129A variant was shown to be insoluble, indicating its role in maintaining the right protein folding of YuiC. The remaining three variants resulted in soluble proteins but were inactive on zymograms indicating that they may be responsible for catalysis. *B. subtilis* cells harbouring individual *sps* genes (*yuiC*, *yabE*, *yocH* and *yorM*) knocked out showed stationary phase defects and altered colony morphologies compared to the wild type.

Conclusion: This study has identified the key residues involved in catalysis of YuiC, which are the D151, D162 and K102. These are conserved in Sps domains. The catalytic mechanism of YuiC is similar to the mechanism reported for *Neisseria gonorrhoeae* MltA. s*ps/yuiC* knock outs have implied that each *sps/yuiC* has a significant role on *B. subtilis* late growth stage.

Significance and Impact of study: The *B. subtilis* YuiC/Sps model has given an insight into Sps functions in the final growth stage of the Firmicutes, which members include etiologic agents of anthrax, botulism and listeriosis. Inhibition of Sps protein may inactivate pathogen replication and facilitate entrance into a non-contagious dormant sporulation stage.

Keywords: Stationary phase survival (Sps), lytic transglycosylase, Firmicutes, sporulation, codon substitution

Introduction

Resuscitation promoting factors (Rpfs) are small peptidoglycan hydrolases with a hybrid of mini lysozyme and lytic transglycosylase fold (Cohen-Gonsaud *et al*., 2005). Rpfs cleave N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) within the peptidoglycan strand. They promote bacterial growth and resuscitate bacterial dormant cells into actively growing cells. However, Rpfs are strictly confined to Actinobacteria including *Mycobacterium tuberculosis*.

Based on the N-terminal domain sequence of *Mycobacterium tuberculosis* resuscitation promoting factor B (*rpfB*), a homologue, the *yabE* gene from *Bacillus subtilis* was found (Ravagnani *et al*., 2005). It was the first gene related to the *rpfs* found in the Firmicute phylum. Although *yabE* has a similar N-terminal domain architecture to *rpfB* comprising of a G5 domain and three DUF348 domains, which are associated with peptidoglycan binding function, its C-terminal *sps* domain was identified from its sequence as a member of a different peptidoglycan hydrolase domain family from the *rpf* catalytic domain. Analysis of their genomic context has demonstrated similar gene arrangement within the operon (Ravagnani *et al*., 2005). This suggests analogy in functionality between RpfB and YabE proteins in cell-wall peptidoglycan remodeling (Ravagnani *et al*., 2005). Ravagnani *et al*. (2005) proposed that Sps proteins are responsible to prolong bacterial survival in the stationary phase prior to entering a sporulation stage. A search based on the *sps* domain sequence at the C-terminal of *yabE* in the Firmicute genomes has found many Sps proteins exist in various bacteria, including the etiologic agents causing anthrax, tetanus and botulism. Apart from *yabE*, three other *sps* genes are also found in *B. subtilis*, *yocH, yuiC* and *yorM*, although *yorM* is a *B. subtilis* prophage derived gene (Ravagnani *et al*., 2005). Expression of *yocH* has been shown to be autoregulated by the presence of muropeptides bound to PrkC kinase and YycFG two-component system (Shah *et al*., 2010). YocH digests peptidoglycan fragments released by its own or other bacteria (Shah *et al*., 2010). Deletion of *B. subtilis yocH* reduced the survival of the bacteria in stationary phase but this effect could be rescued by supplementation of YocH at very low concentrations (Shah *et al*., 2010). Deletion of *Listeria monocytogenes* Sps proteins has lengthened the bacterial lag phase growth in minimal medium but addition of the corresponding recombinant proteins was able to stimulate the growth of the bacteria (Pinto *et al*., 2013). The dormant cells of *Staphylococcus aureus* have also been shown to be revived by addition of spent culture supernatant from cultures of the same organism

(Pascoe *et al*., 2014). The structure of *B. subtilis* YuiC has been solved at 1.2 Å resolution and showed that it is a minimal version of the membrane bound lytic transglycosylase A (MltA) of *Escherichia coli* (Quay *et al*., 2015). Structural comparison of the *E. coli* MltA Domain A and the ordered β-barrel domain of YuiC (P73-E218) matches only to the Domain A of *E. coli* MltA and the core domain of YuiC is 33 residues shorter than the *E. coli* MltA (Quay *et al*., 2015). In this paper, some of the conserved residues in Sps domains were mutated to study the key residues involved in muralytic catalysis. The effect of *sps* genes knockout on the *B. subtilis* cell growth was also studied.

Materials and Methods

Site-directed, Ligase-Independent Mutagenesis Polymerase Chain Reaction (SLIM PCR)

A truncated *yuiC* construct, R52-E218-pNIC28-BSA4 plasmid was used (Quay *et al*., 2015). Point mutations D129A, D151A, D162A and K102A were selected based on the identification of three active site aspartic acid residues (D129, D151 and D162) from the NCBI Conserved Domain Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) using the *yuiC* amino acid sequence. The coordinates of these three aspartic acid residues were also examined on the YuiC three-dimensional structures (PDB ID 4wjt, 4wli and 4wlk). K102 was selected as it had potential interactions for the bound anhydrodisaccharide sugar. Forward and reverse primers for introducing point mutations were generated using PrimerX tool (http://www.bioinformatics.org/primerx/). The sequences of the forward and reverse primers designed for each of the mutations are listed below:

D129A_F (5’GTTGCTGCTGCGCCGTCCGTTTTCCCGATCGGAACAATTTTATTC3'),

D129A_R (5’AAAACGGACGGCGCAGCAGCAACCGTGGAATATAAATCCCGTTTCA3'),

D151A_F (5’GTTGTGGCCGCGACAGGATCAGCTATTAAGGGAAACCGC3'),

D151A_R (5’CTGATCCTGTCGCGGCCACAACTCCGAGACCGTAGTTCG3'),

D162A_F (5’AAACCGCCTTGCGCTGTACTTTGAGACAGTCAAAGATGTATAC3'),

D162A_R (5’TCAAAGTACAGCGCAAGGCGGTTTCCCTTAATAGCTGATCCTG3'),

K102A_F (5’GAGTCGACGGGCGCGAATCCCGGTGATCCATTATACGGGCTTAC3'),

K102A_R (5’ATCACCGGGATTCGCGCCCGTCGACTCAGCTCCTGCTGTGTAGC3').

SLIM PCR was employed in this work as it is a simple method to introduce mutations in a gene by amplification using two primers that enable the production of an entire plasmid without the need of restriction site manipulations. Primers that contain the desired substitution are designed with long 3’ overhangs such that both forward and reverse primers will be annealed complementarily to the template.

Polymerase Chain Reaction (PCR)

Amplification of the entire plasmid containing the gene of interest with desired mutation was performed in a 25 μL reaction volume containing 0.4 μM forward primer (1 μL), 0.4 μM reverse primer (1 μL), 5% DMSO (1.25 μL), 70 ng/μL template DNA (0.3 μL), 2X KAPA HiFi (1X, 12.5 μL) and ddH_2O (8.95 μL). The annealing temperatures tested were 58°C, 60°C and 63°C. The final conditions for the PCR cycle performed were 95°C for 5 min (1 cycle), 98°C for 20 s, 60°C for 15 s and 72°C for 6 min (30 cycles) and 72°C for 6 min (1 cycle).

Cell growth and protein expression

After PCR amplifications, the size of the plasmids was examined via agarose gel (1%, w/w) electrophoresis. The plasmids were sequenced with T7 forward and reverse primers by GATC Biotech, UK. The sequence of the plasmids was checked using Chromas software

(Technelysium, Australia) and ClustalW (Larkin *et al*., 2007), to confirm the site directed mutagenesis of the four constructs. The sequenced plasmids were transformed into *E. coli* BL21 (DE3) competent cells. Protein expression was begun by performing starter cultures for each of the colonies for the four variants, each in 100 mL LB media supplemented with 50 µg/mL Kanamycin (Kan) and 34 µg/mL Chloramphenicol (Cam). The starter cultures were incubated overnight at 37°C with shaking at 180 rpm. Protein expression was carried out according to the methods described by Quay *et al*. (2015). Starter cultures were inoculated into each 500 mL Terrific Broth (TB) supplemented with Kan (50 µg/mL) and Cam (34 µg/mL) to achieve an OD_{600} of 0.02. Protein induction was initiated by adding 0.25 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) into each flask when the cultures have reached OD_{600} 0.4-0.6. The cultures were further incubated for 6 hours at 37°C, 180 rpm. The cells were harvested by centrifugation at 822 x g at 4°C and stored at -20°C.

Protein purifications

Cells lysis and protein purifications were performed according to the methods described by Quay *et al*. (2015). Briefly, the harvested cells were mixed with lysozyme (1 mg/mL) and treated by sonication (Sonics, Vibra Cell). The cell suspension was centrifuged at 48,000 x g for 60 minutes at 4°C to discard the cell pellet. The supernatant that contains the His-tagged YuiC variants were purified via three steps of chromatography which are immobilised metal affinity chromatography, cation exchange chromatography and size exclusion chromatography as described in Quay *et al*. (2015). Protein elution was monitored by the UV absorbance at 280 nm. Samples were collected and analysed at each stage by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) stained using Instant Blue (Expedeon) and Western blot using the iBlot device with rolling blotter (Invitrogen).

Zymogram

A zymogram was prepared by adding 0.2% (w/v) *Micrococcus lysodeikticus* (lyophilised *M. luteus* cells) (Sigma-Aldrich) into the 12.5% SDS-PAGE gel. The components used for making the 0.75 mm 10% stacking SDS-PAGE gel for zymogram were 30% (w/v) Acrylamide Ratio 37.5:1 (Severn Biotech) (415 µL), 1.0 M Tris-HCl (pH 6.8) (315 µL), 10% SDS (w/v) (25 µL), 10% APS (w/v) (25 µL), TEMED (2.5 µL) and ddH_2O (1.7 mL). The components used for making 0.75 mm 12.5% resolving SDS-PAGE gel for zymogram include 30% (w/v) Acrylamide Ratio 37.5:1 (Severn Biotech) (2.083 mL), 1.5 M Tris-HCl (pH 8.8) (1.25 mL), 10% SDS (w/v) (50 µL), 10% APS (w/v) (50 µL), TEMED (2 µL), ddH_2O (1.565 mL) and 0.2% (w/v) *M. lysodeikticus* (80 mg). Prior to loading the protein samples into the zymogram gel, 3 µL of 4X SDS loading buffer were mixed with the samples without β-mercaptoethanol (βME) and the protein samples were not heated. The electrophoresis was run at 180 V for 60 minutes. The gel was washed three times in 100 mL ddH_2O for 20 minutes to remove the SDS. Then, 100 mL renaturation buffer (50 mM sodium phosphate (pH 5.0) and 1% (v/v) Triton X-100) was then added onto the gel and rinsed for 30 minutes. The gel was incubated in fresh renaturation buffer for two days. Zymogram staining was performed with 0.1% methylene blue in 0.01% potassium hydroxide and de-stained in ddH_2O until clear bands were visible against the blue gel background.

***B. subtilis* Sps mutant cell growth**

B. subtilis mutants with the s*ps* genes (*yuiC, yocH, yabE* and *yorM*) knocked out with an erythromycin cassette (Koo *et al*., 2017; Yan *et al*., 2008) were purchased from the BGSC

(Bacillus Genomic Stock Centre). Colony PCR and DNA sequencing were done for each mutant to confirm the respective gene knockout. The sequences of the primers used for colony PCR are listed below.

YuiC_KO_F 5' CGGAGAGCTTGAGGAATTC 3',

YuiC_KO_R 5' CGTATCAAGCAGAACAGCAG 3',

YorMKO_F 5' TCTTGGTATACCAGAGGAACA 3',

YorMKO_R 5' CCAGTTGATTTTACCTTAGCC 3',

YabEKO_F 5' GAGCTTGTCCCTTACAGCG 3',

YabEKO_R 5' GCCCCTCGACCACAATGAT 3',

YocHKO_F 5' TTCAGCAAGTCTTTCCATCAG 3',

YocHKO_R 5' GAGCCCCTGAATCGCACAT 3'.

The conditions used for the colony PCR cycles were 95°C for 10 min (1 cycle), 98°C for 20 s, 60°C for 15 s and 72°C for 6 min (30 cycles) and 72°C for 6 min (1 cycle). The mutants were streaked on LB agar and incubated overnight at 37°C. The morphology of the colonies was monitored after 18 hours. Bacterial cultures were grown in buffered LB without salt, pH 6.9 at 37°C (Gaidenko *et al*., 2002; Shah *et al*., 2010). Cell growth was monitored at OD_{600} for a period of 45 hours. The growth curve was measured twice at the indicated time points.

Results and Discussion

Point Mutation Studies of *yuiC* via SLIM PCR

Four individual *yuiC* constructs with the desired substitutions were generated via SLIM PCR. The substitutions are D129A, D151A, D162A and K102A. D129, D151 and D162 were selected based on the identification of the conserved aspartic acid residues on the NCBI conserved domain search server and the coordinates of the YuiC-ligand complex three-dimensional structure (Quay

et al., 2015). These residues are also conserved within the Sps proteins among the Firmicutes (Ravagnani *et al*., 2005). D308 in *E. coli* MltA, a residue analogous to D162 in YuiC, has been identified as the catalytic residue. Mutation of this residue in *E. coli* MltA has completely abolished the enzymatic activity (van Straaten *et al*., 2007). Quay *et al*. (2015) has shown that the K102 forms a hydrogen bond interaction with the ligand NAG at the -2 sugar position, but this residue does not seem to be conserved among the Rpfs and MltA protein (Ravagnani *et al*., 2005). Hence, this may be a unique interaction for the YuiC/Sps catalysis. The *yuiC* variants with the expected product size of ~5.6 kb was successfully amplified at 58°C, 60°C and 63°C after annealing temperature optimisations (Figure 1).

Protein Expression and Purification

YuiC D129A, D151A, D162A and K102A constructs were heterologously expressed using *E. coli* BL21 (DE3) cells and purified to homogeneity using three-step chromatography as described in Quay *et al*. (2015). SDS-PAGE (Figure 2A) and Western blot analysis (Figures 2B) confirmed that the D129A construct produced an insoluble YuiC variant but the other three constructs (D151A, D162A, and K102A) were expressed as soluble proteins. D129 is not close to the ligand (Quay *et al*., 2015), indicating that it has an essential role in maintaining the folding of the protein but not in catalysis. YuiC proteins tend to form monomeric and dimeric proteins (Quay *et al*., 2015). The crystal structure of YuiC complex (PDB ID 4wlk) has shown the presence of a hydrolysed ligand, the anhydrodisaccharide sugar molecule bound in the protein, implying that the dimeric protein of YuiC is an active form but the activity of the monomeric protein was yet to be confirmed (Quay *et al*., 2015). D162A variant was also exist in two forms which is observed as two peaks on size exclusion chromatography, corresponding to the monomer and dimer respectively (Supplementary Figure 1).

YuiC Catalysis via Zymogram Analysis

In order to assess the enzymatic activity of YuiC variants, the three soluble mutant constructs (D151A, D162A and K102A) were analysed via zymography. The zymogram was prepared using SDS-PAGE gel copolymerized with 0.1% of *M. lutues* lyophilized cells as the source of peptidoglycan substrate. Peptidoglycan is made up of strands of alternating NAG and NAM molecules cross-linked to peptides. Gram positive bacteria have thicker peptidoglycan layers (may contain up to 40 layers), contributing to about 20% of the cell wall dry weight. During gel staining, the methylene blue dye will bind to the peptides within the peptidoglycan copolymerized with SDS-PAGE gel. When the enzymatic reaction takes place, the active enzyme will cleave the β-1,4-glycosidic bonds between NAG and NAM. The peptidoglycan strands will be fragmented and diffused out of the gel. A clear zone will be seen against the blue background surrounding the protein band on the zymogram. The protein samples used in the zymogram were the purified proteins of YuiC wild type (WT) and D162A but crude lysates of D151A and K102A. YuiC WT and D162A monomeric and dimeric proteins were obtained from different size exclusion chromatography fractions (Quay *et al*. 2015).

Although it did not photograph well, the zymogram (Figure 3B) run together with the SDS-PAGE (Figure 3A) showed that D151A (lane 2) and K102A (lane 4) both contained inactive enzymes in the lysate and cell pellet. The purified YuiC WT proteins (lanes 6, 12 and 13) were active as white bands were shown against the background. The negative controls (lanes 7, 8 and 14) showed no catalytic activity. The D162A monomer (lane 10) and dimer (lane 11) both showed no activity. This implies that the residues D151, D162 and K102 are involved in catalysis. These observations corroborate with the structural analysis that D151, D162 and K102

are forming hydrogen bonds interactions for catalytic activity in the presence of ligand (Quay *et al*., 2015).

Structural comparison between *E. coli* MltA (PDB ID 2ae0, amino acids 20-104 and 243-337) and YuiC has a RMSD of 2.14 Å/98 Cα but the catalytic mechanism may not be similar between YuiC and MltA (Quay *et al*., 2015). The *E. coli* MltA catalysis involves one catalytic aspartic acid residue and helix dipole electrostatic interaction for the stabilization of the oxocarbenium ion transition state but this mechanism may not be possible for YuiC because YuiC has a shorter equivalent α-helix (van Straaten *et al*., 2005, Quay *et al*., 2015). Structural comparison with *Neisseria gonorrhoeae* MltA (PDB ID 2g5d) (Powell *et al*., 2006) has found that D151 and D162 of YuiC are located at an equivalent position to the D393 and D405 of MltA respectively. *N. gonorrhoeae* MltA reported a different mechanism involving two carboxylic acids (D393 and D405) as the catalytic residues (Powell *et al*., 2006). The RMSD between the core domain of *N. gonorrhoeae* MltA (PDB ID 2g5d, amino acids 40-142 and 343-441) and YuiC (PDB ID 4wjt, chain A: 72-176, chain B: 177-217) is 1.97 Å/91 Cα. Our mutation studies have shown that both D151 and D162 are important for YuiC catalytic activity. This implies that the catalytic mechanism of YuiC is similar to the one proposed by *N. gonorrhoeae* MltA (Powell *et al*., 2006). This leads to the proposal that the YuiC protein catalysis involves the D151 residue donating a proton to the oxygen atom (O) between the NAG molecules to cleave the β-1,4 glycosidic bond and form an oxocarbenium ion (Figure 4). As the oxocarbenium ion is an unstable transition state, D162 will then act as a base to abstract a proton from the C6OH to generate nucleophilic attack on the C1 atom to form a more stable 1,6-anhydrosugar. Y93 of YuiC which is located in between the D151 and D162, also has an equivalent position to the *N. gonorrhoeae* MltA Y140, indicating that this residue is responsible for shuttling a proton from D162 to D151 to regenerate its protonation state.

On the other hand, K102 in YuiC is not conserved among the Rpfs and MltA (Ravagnani *et al*., 2005) but this residue makes a hydrogen bond interaction with the NAG (O3A) at -2 position (Quay *et al*., 2015). This is presumably important for stabilizing the ligand bound in the Sps domain by properly orienting the sugar molecules for more efficient catalysis. Both NAG and the 1,6-anhydrosugar has shown to bind K102 at the -2 sugar position in YuiC protein complex (Quay *et al*., 2015) indicating that this interaction makes a strong anchoring point to YuiC catalysis. YuiC requires more hydrogen bond interactions compared to other homologous lytic transglycosylases. Mutation of this residue weakened the binding of the ligand in the active site, reducing the catalytic activity in YuiC.

Phenotypic Evaluation of Δ*sps* Mutants of *B. subtilis*

The YuiC mutagenesis work is further supported by the microbiological observations that deletion of any of the s*ps* genes exerted a clear effect on the phenotype of the *B. subtilis* cells particularly on its stationary phase. The Bacillus Genomic Stock Centre (BGSC) has a library of 4000 mutants with knockout strains including the *sps* genes, *yocH, yabE, yorM* and *yuiC*. Those mutants were purchased to investigate their phenotypic changes. These *sps* knockout mutants have been confirmed via colony PCR (Figure 5) and DNA sequencing (Koo *et al*., 2017, Yan *et al*., 2008, http://genolist.pasteur.fr/SubtiList/). The erythromycin resistant gene cassette is 900 bp (Koo *et al*., 2017, Yan *et al*., 2008). Upon replacement, *sps* genes smaller than the erythromycin resistant gene cassette will result in bigger amplicon band size than the wild type and vice versa. The mutant cassettes for *yocH* (wild type: 861 bp), *yorM* (wild type: 714 bp), *yuiC* (wild type: 654 bp) and *yabE* (wild type: 1311 bp) will be 39 bp bigger, 186 bp bigger, 246 bp bigger and 411 bp smaller than the wild type *yuiC* construct respectively. Therefore, the expected size of the PCR products for *yuiC*, *yorM*, *yabE* and *yocH* knocked out mutant strains are 2892 bp, 1245 bp,

1211 bp and 1180 bp respectively compared to the wild type 2646 bp, 1059 bp, 1622 bp and 1141 bp respectively (Figure 5).

The mutants were grown on LB agar, incubated at 37°C for 18 hours and the morphology of the colonies was monitored. All the individual Δ*sps* mutants demonstrated a significant difference in their colony morphologies compared to the wild-type *B. subtilis* strain 168. The wild type strain had an uneven-edged colony shape which is termed lobate, whereas the mutants had a much rounder and even-edged colony shape (Figure 6). *B. subtilis* wild type strain moves and grows on solid media via swarming motility as the flagellated bacteria migrate in a group (Calvio *et al*., 2005). A rounded colony edge indicates that the swarming effect is restricted which would cause reduced cell growth.

On the other hand, the effect of each of the Sps mutants has been assessed by their growth curves (Figure 7). Powell *et al*. (2006) has shown a reduced cell growth for YocH mutant at the stationary phase. In this work, we included three other paralogues of *yuiC* (*ΔyocH, ΔyabE* and *ΔyorM*) in the deletion studies. All mutants showed defect in the stationary phase in which the mutant strains died quicker than the wild type strains. Of the three intrinsic genes, *ΔyocH* exhibited the most significant effect, followed by *ΔyabE* and *ΔyuiC*. *yorM* is an *sps* from the SPβ prophage that is usually infects *B. subtilis*. It exhibited the strongest effect among all four *sps*, indicating a survival advantage for incorporating the prophage.

This preliminary result showed that each of the four *sps* genes including *yorM* has shown that they are indispensable for stationary phase survival in *B. subtilis.* All this evidence have demonstrated the specific function of Sps proteins particularly on reviving stationary phase cells, analogous to the function of Rpfs which resuscitate dormant mycobacteria cells.

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Figures

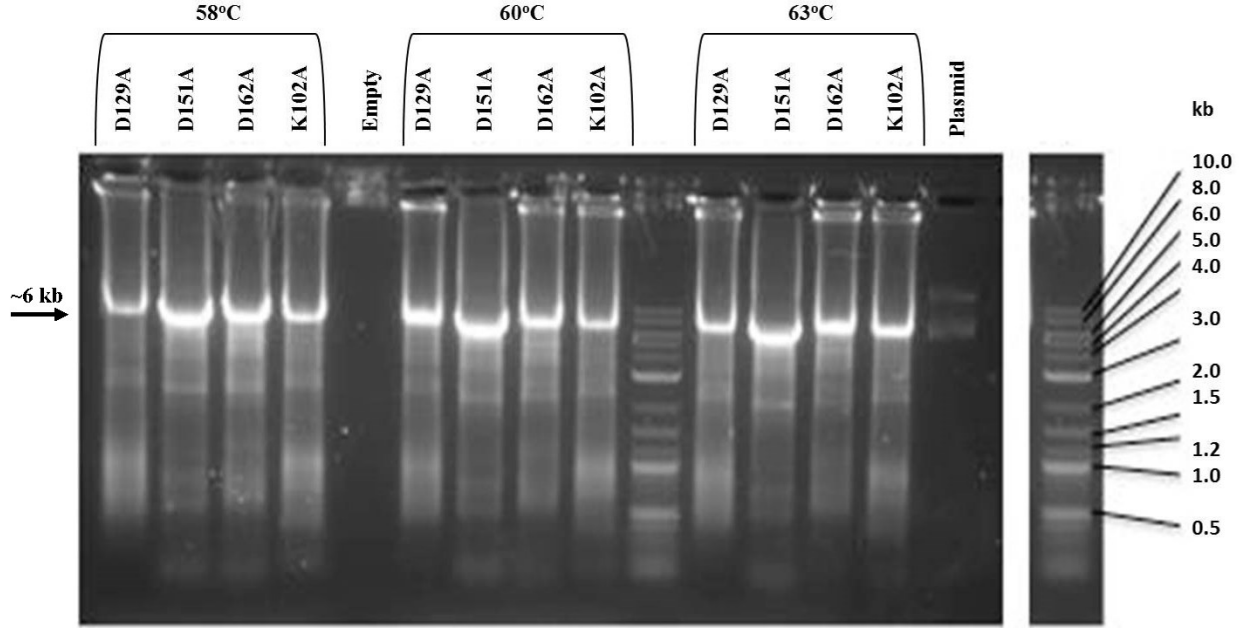

Figure 1. Agarose gel (1% w/w) electrophoresis profile shows the amplified *yuiC* variants at 58°C, 60°C, and 63°C for the four forms of *yuiC* mutation (D129A, D151A, D162A and K102A) in pNIC28-BSA4 plasmid respectively.

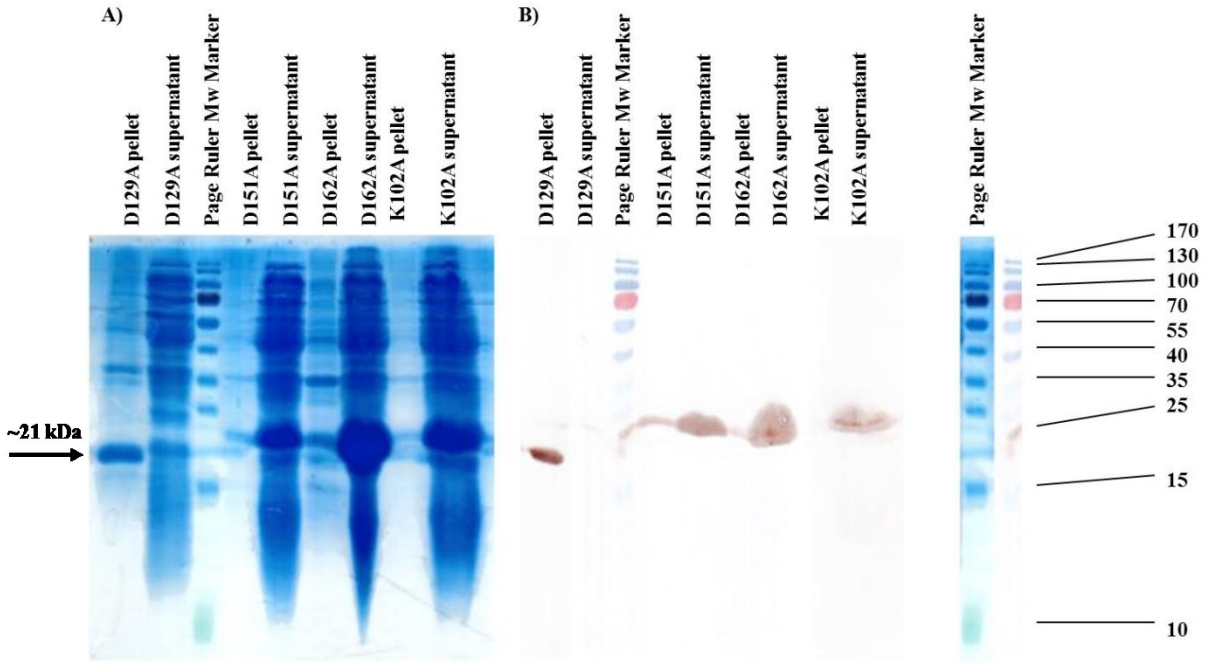

Figure 2. (A) SDS-PAGE (12.5%) and (B) Western blot profile of the pellet and supernatant of the four YuiC variants expressed in *E. coli* BL21 (DE3) cells.

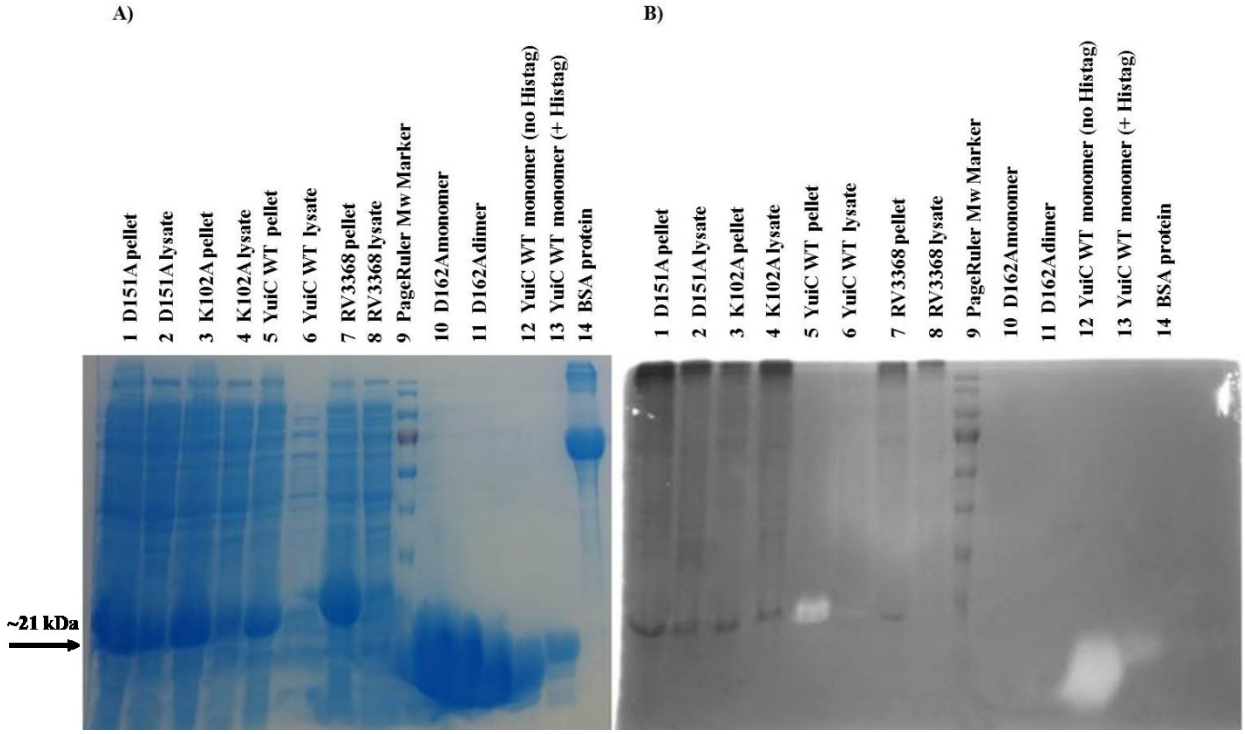

Figure 3. (A) SDS-PAGE (12.5%) gel run with the zymogram (B) Zymogram profile shows the YuiC variants were catalytically inactive compared to the wild type (WT). YuiC wildtype (WT) was used with and without the His-tag as positive controls, RV3368 (a putative nitroreductase from *Mycobacterium tuberculosis*) and BSA were used as negative controls.

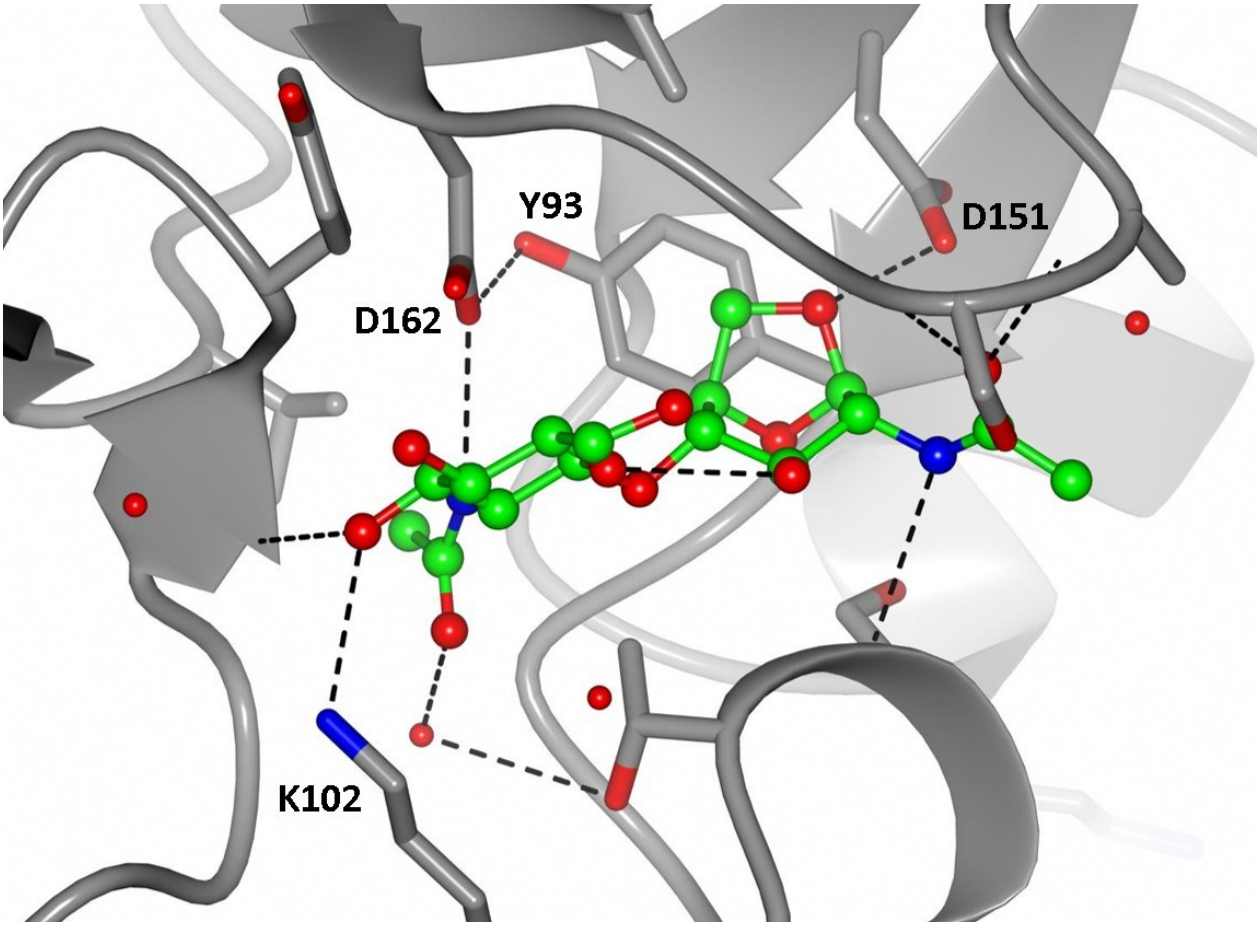

Figure 4. Interactions between anhydrodisaccharide sugar molecule with *B. subtilis* YuiC showed with hydrogen bonds (PDB ID 4wlk). D151, D162 and Y93 are responsible for catalysing YuiC lytic transglycosylase activity similar to the *N. gonorrhoeae* MltA. D151 and D162 are acting as the catalytic carboxylate residues for donating and abstracting protons respectively to cleave β-1,4-glycosidic bond and initiate nucleophilic attack to form 1,6-anhydro-murosugars. Y93 is responsible for transferring a proton between the two carboxylic acids to regenerate the protonation state. K102 orients the sugar molecules for efficient catalysis. YuiC protein structure is shown in grey broken chain with the ligand binding residues. Anhydrodisaccharide sugar molecule is shown in balls and sticks representation. Hydrogen bonds are shown in black dashed lines. Water molecules are shown as red balls. Green, red and blue color on the protein chain and anhydrodisaccharide sugar molecule denote carbon, oxygen and nitrogen atoms respectively.

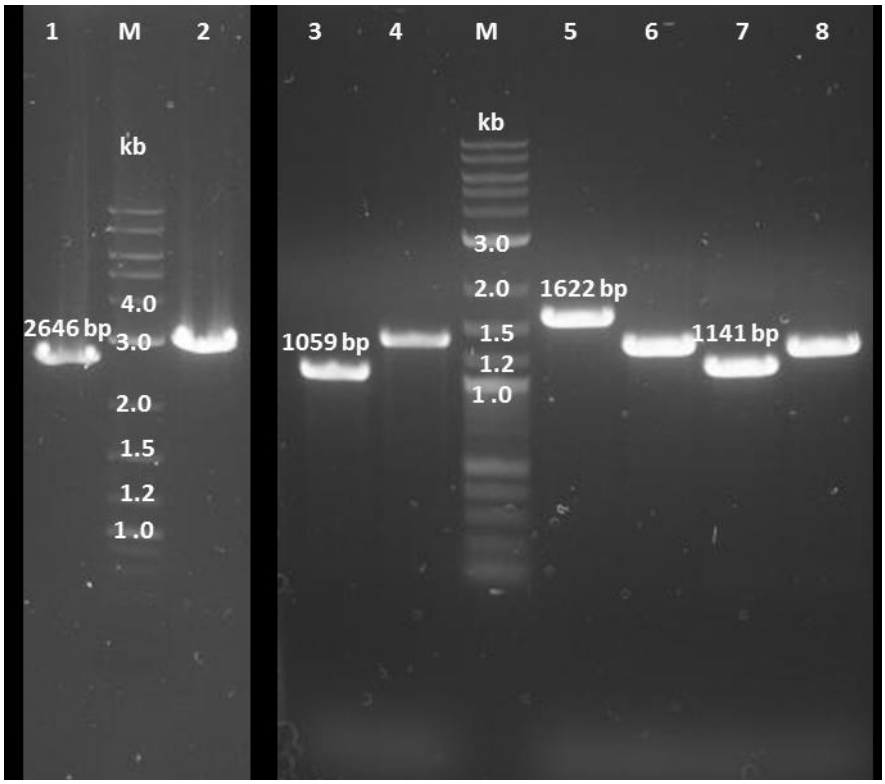

Figure 5. *B. subtilis sps* gene knockouts verification by colony PCR. Each pair of primers flanking the start and the stop codon of *yuiC, yorM, yabE* and *yocH* respectively was used to amplify the targeted region via colony PCR. The agarose gel (1%, w/w) profile shows the amplicons from colony PCR using *B. subtilis* 168 wild type and Δ*sps* mutant strains. Lane 1: wild type; M: 2-Log DNA Ladder (0.1-10.0 kb, NEB); 2: Δ*yuiC* mutant; 3: wild type; 4: Δ*yorM* mutant; M: 2-Log DNA Ladder (0.1- 10.0 kb, NEB); 5: wild type; 6: Δ*yabE* mutant; 7: wild type; 8: Δ*yocH* mutant. The deletion cassette is 246 bp bigger than *yuiC*, 186 bp bigger than *yorM*, 411 bp smaller than *yabE* and 39 bp bigger than *yocH*. The marked sizes are the spacing of the primers in the wild type strain.

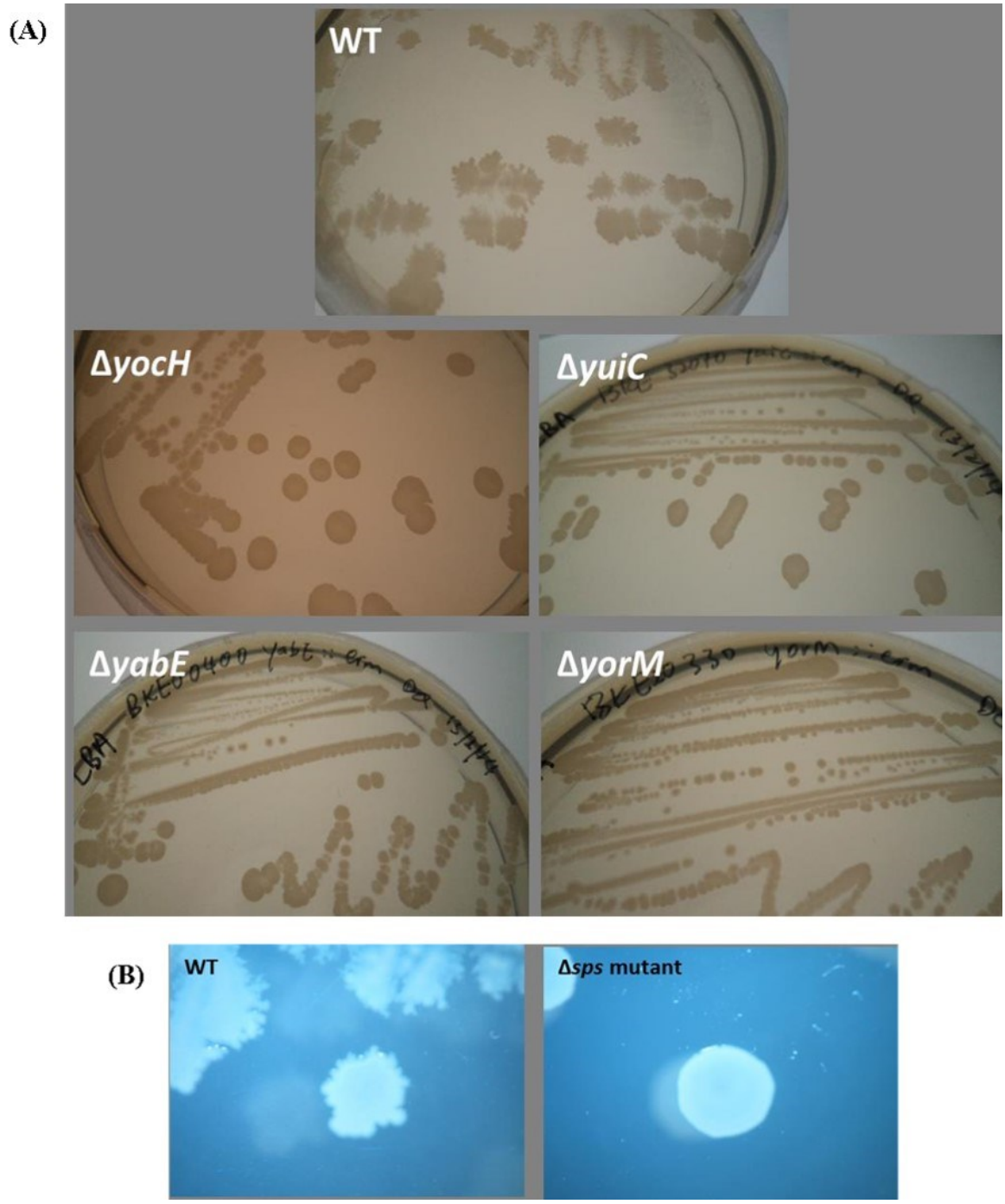

Figure 6. (A) Colony morphology of the *B. subtilis* wild type (WT) and the *sps* mutant (Δ*yocH*, Δ*yuiC*, Δ*yabE* and Δ*yorM*) strains. *B. subtilis* 168 wild type strain and its *sps* mutant strains were streaked on LB agar plate and incubated at 37°C for 18 hours. (B) Close up view at 10x magnification of the wild type and one of the mutant colonies (Δ*yuiC* mutant) were taken by a

digital camera attached on a dissecting microscope (Zeiss). Wild type colonies have an uneven colony edges whereas the mutants have more rounded colony edges.

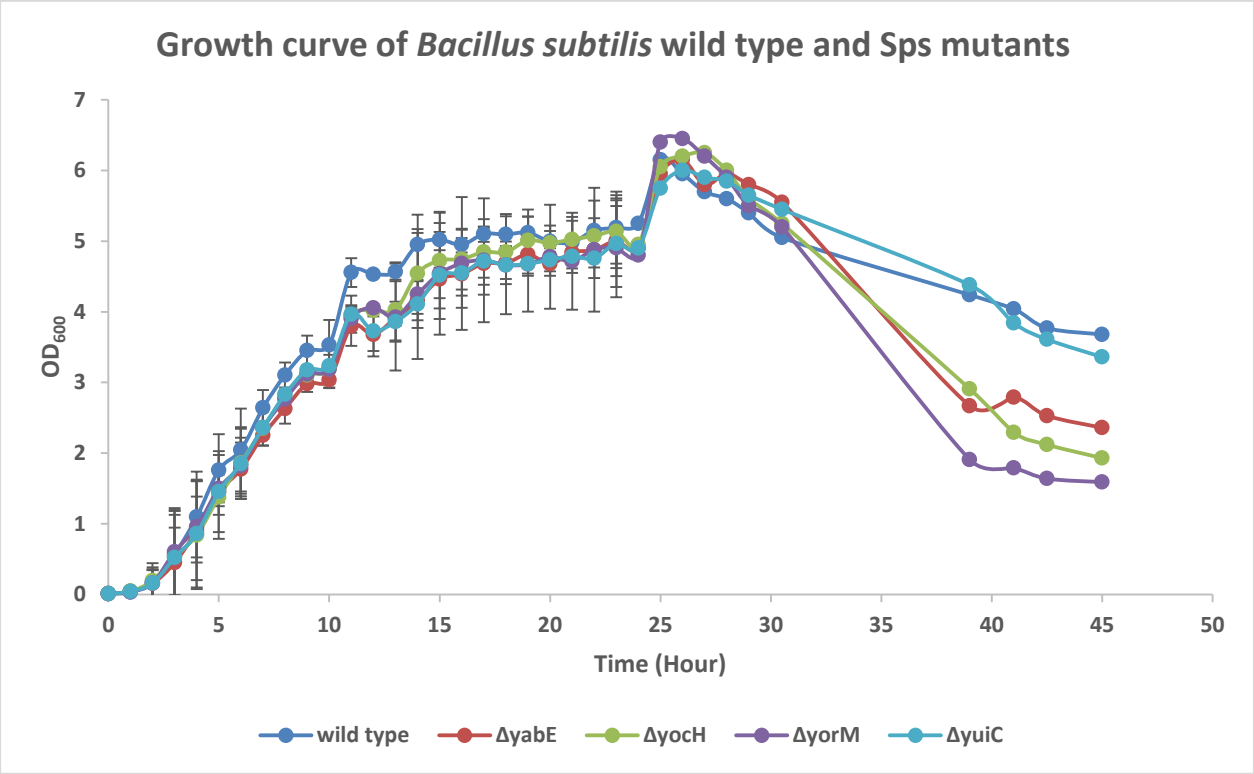

Figure 7. Absence of each *sps* gene in *B. subtilis* reduces the survival at the stationary phase. Absorbance OD_{600} was measured for 45 hours at the indicated intervals.