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In Silico Analysis and Validation of Ymca Gene of Bacillus Subtilis

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Abstract

This study aimed to analyze and validate the ymcA gene of *Bacillus subtilis* in silico. The bacterial strains used were *B. subtilis* 2049 (T) and *B. subtilis* HS, obtained from the National Center for Microbial Resource, Pune. The media used were LB medium and Mineral Salt Medium (MSM). The biofilm formation assay was performed in a microtiter plate, with each well inoculated with 980 µl of MSM and 20 µl of test cultures. The plate was incubated for 120 hrs at 37°C following phosphate buffer assay. The contents of the plate were removed by gently tapping at the bottom of the plate, rinsed with phosphate buffer saline, and de-stained using 70% ethanol for 15 min. The genomic DNA of *B. subtilis* 2049 (T) was extracted using the Chloroform: Isoamylalcohol method. The supernatant was taken in a sterile tube and stored at -80°C until further use.

Keywords: In Silico Analysis, ymcA Gene, *Basillus subtilis*

1. Introduction

1.1 *Bacillus subtilis:* Biofilm forming bacteria

This study aimed to analyze and validate the ymcA gene of *Bacillus subtilis* in silico. The bacterial strains used were *B. subtilis* 2049 (T) and *B. subtilis* HS, obtained from the National Center for Microbial Resource, Pune. The media used were LB medium and Mineral Salt Medium (MSM). The biofilm formation assay was performed in a microtiter plate, with each well inoculated with 980 µl of MSM and 20 µl of test cultures. The plate was incubated for 120 hrs at 37°C following phosphate buffer assay. The contents of the plate were removed by gently tapping at the bottom of the plate, rinsed with phosphate buffer saline, and de-stained using 70% ethanol for 15 min. The genomic DNA of B. subtilis 2049 (T) was extracted using the Chloroform: Isoamylalcohol method. The supernatant was taken in a sterile tube and stored at -80°C until further use.

1.2 *ymcA* gene

The ymcA gene, a part of the PSPI family, governs the biofilm formation ability of many organisms, including *B. subtilis*. During environmental stress, the ymcA gene forms the ymcA protein, which is responsible for biofilm assembly and maturation. The ymcA gene inactivates the repressor of biofilm formation gene sinR, preventing biofilm formation. YlbF and ymcA are essential proteins for biofilm formation, sporulation, and competence in *Bacillus subtilis*. The ymcA gene is a subunit of the regulatory iron-sulfur containing ymcA-ylbF-yaaT complex, required for RNase Y dependent maturation of polycistronic mRNAs. During environmental stress, *Bacillus subtilis* undergoes developmental changes leading to biofilm formation, sporulation, and competence. The phosphorylated

form of the master response regulator Spo0A (Spo0A~P) accelerates the phosphorelay and helps the organism in biofilm formation.

1.3 Biofilm formation in *B. subtilis* using *ymcA* gene

Biofilm formation in *Bacillus subtilis* is a complex process involving the expression of the ymcA gene. This gene plays a crucial role in the formation of structured multicellular biofilms, which protect cells from external stress and provide an environment for gene transfer and quorum sensing. The ymcA protein, a dimer consisting of two chains, belongs to the Com_YlbF superfamily and is essential for correct biofilm formation. The ymcA protein is responsible for the formation of robust biofilms, which are mainly composed of polysaccharides. The EPS, which makes up nearly 50% to 90% of the total organic carbon responsible for biofilm formation, varies in physical and chemical properties. The Com_YlbF superfamily includes both ylbF and ymcA, which can interact synergistically. Null mutants of ymcA and ylbF fail to form pellicles at air-liquid interfaces and grow on solid media as smooth, undifferentiated colonies. These findings have broad implications for various bacterial species.

2. Method Used

2.1 Bacterial strains and Media used.

B.subtilis 2049 (T) and *B. subtilis* HS were the bacterial strains employed in this investigation. Solidified agar, Mineral Salt Medium (MSM), and LB medium were among the media used. After drying the medium at 25°C, the presence of bacteria was examined.

2.2 Biofilm development in *B.subtilis 2049 (T)* and *B. subtilis HS.*

The biofilm formation assay involved inoculating a culture in LB Broth, incubating it for 24 hours, centrifuging it, discarding the supernatant, and resuspending the pellet in Minimal Salt Medium. The process involved a range of chemical elements and pH levels.

The pellet was mixed, and microtiter plates were inoculated with MSM and test cultures. The plates were incubated for 120 hours at 37°C. After removal, the plates were rinsed with phosphate buffer saline, stained with Crystal Violet, and incubated at room temperature for 45 minutes. The optical density was measured using spectrophotometric analysis.

2.3 Genomic DNA isolation

The genomic DNA of *B. subtilis* 2049 (T) was extracted using the Chloroform: Isoamylalcohol method. The cultures were inoculated in LB medium and incubated at 37°C until an optical density of 1 OD was reached. The supernatant was discarded, and the pellet was resuspended in TE buffer pH 8.0, added with SDS, Proteinase k, and Lysozyme. The tubes were then incubated in a water bath at 65°C for 10 minutes, followed by 200 µl of chloroform: Isoamyl alcohol, centrifuged at 10,000 rpm, and incubated overnight in the refrigerator. The content was centrifuged at 10,000 rpm for 15 minutes, and the supernatant was discarded. The pellet was air dried in an incubator for 2-3 hours, resuspended in TE buffer, and further analyzed by gel electrophoresis.

2.3.1 Purity of genomic DNA

The DNA concentration is determined by measuring absorbance at 260nm, adjusting for turbidity, multiplying by the dilution factor, and using the relationship that an A260 of 1.0 equals 50 µg/ml pure dsDNA. The purity of the DNA sample is checked by taking absorbance at 260nm and 280nm, and the OD is divided.

2.3.2 Gel Electrophoresis.

The agarose gel electrophoresis involved preparing a 1% agarose gel by dissolving agarose powder in TAE buffer and heating it. DNA samples of *B. subtilis* 2049 (T) and *B. subtilis* HS were loaded into the gel using bromophenol blue, and the electrophoretic analysis was performed using 1% agarose gel and 1x TAE (Tris acetate-EDTA). The gel was run at 80 volts for 10-15 minutes until DNA was separated by molecular weight. The gel was visualized under UV spectrophotometer.

2.4 Detection of *ymcA* in *B. subtilis 2049 (T) and B. subtilis HS*

The ymcA gene sequence of *B. subtilis* strains was analyzed using the National Center for Biotechnology Information (NCBI) gene database. Multiple Sequence Alignment (MSA) was performed using the ClustalW tool to identify gene variation within the strains. Primers were designed using Primer3 tool, with ymcA (5'-TTAGAGAGAACAACAGCTGTTATTTGAATGC-3'R) as the reverse primer and ymcA (5'- ATGACGCTACTCAAAAAAAGAC-3'F) as the forward primer. The isolated genomic DNA of *B. subtilis* 2049 (T) and *B. subtilis* HS was used for ymcA gene amplification. The PCR machine programme was followed, with initial denaturation at 94°C, annealing at 50°C for 1 minute, extension at 72°C for 1.5 minutes, and final extension at 72°C for 10 minutes. The amplified ymcA gene was visualized using a 1% agarose gel with EtBr and loaded with dye-Bromophenol blue.

2.5 Bioinformatics studies of *ymcA* gene of *B.subtilis*

The large scale bioinformatics analysis of the *Bacillus subtlis* and its protein *ymcA*, which is involved in the formation of biofilms was done using the following tools:

2.5.1. Multiple Sequence Alignment

The ymcA gene sequence of *B. subtilis* strains was analyzed using the National Center for Biotechnology Information (NCBI) gene database. Multiple Sequence Alignment (MSA) was performed using the ClustalW tool to identify gene variation within the strains. Primers were designed using Primer3 tool, with ymcA (5'-TTAGAGAGAACAACAGCTGTTATTTGAATGC-3'R) as the reverse primer and ymcA (5'- ATGACGCTACTCAAAAAAAGAC-3'F) as the forward primer. The isolated genomic DNA of *B. subtilis* 2049 (T) and *B. subtilis* HS was used for ymcA gene amplification. The PCR machine programme was followed, with initial denaturation at 94°C, annealing at 50°C for 1 minute, extension at 72°C for 1.5 minutes, and final extension at 72°C for 10 minutes. The amplified ymcA gene was visualized using a 1% agarose gel with EtBr and loaded with dye-Bromophenol blue.

2.5.2. Phylogenetic analysis of the *ymcA* gene

The phylogenetic relatedness of the ymcA protein was determined using the simple phylogeny tool. The phylogenetic tree was generated by performing MSA on the ymcA gene of different *B. subtilis* strains using the ClustalW tool. The.aln file was then used in the simple phylogeny tool search program.

2.5.3. Identification of the conserved domain of the *ymcA* gene

CDD, the Conserved Domain Database, is part of NCBI's Entrez query and retrieval system and is also accessible via http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml which provides the insights of the conserved domains lying in the protein of our interest. CDD provides annotation of protein sequences with the location of conserved domain footprints and functional sites inferred from these footprints (Lu S et al., 2020; Marchler-Bauer et al., 2013). CDD incorporates several protein domain and full-length protein model collections, and maintains an active curation effort that aims at providing fine grained classifications for major and well-characterized protein domain families.

The conserved domain of the following query protein was obtained from the ncbi website through the CDD (conserved domain database) tool following with the same query sequence KIX82597.1 using search filter with concise results with the CDD id: gi/760456882/gb/82597/.

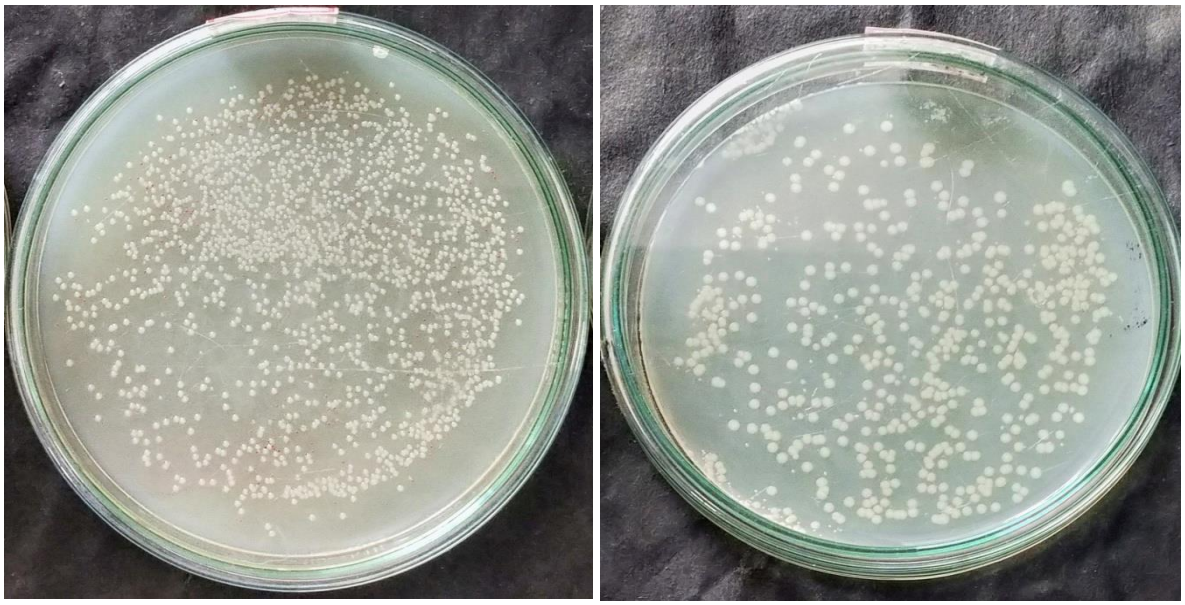
2.5.4. Elucidation of the secondary structure of the *ymcA* protein.

The protein structure of the ymcA protein of *B. subtilis* was obtained from the RCSB PDB database using X-Ray diffraction and Raswin software. This method provides detailed information about the protein's architectural features, atomic arrangements, active catalytic sites, and residue level views. The study aids in understanding the protein's structural properties and potential enzyme activity.

3. Result and conclusion

3.1 Revival of the strains *B.subtilis 2049 (T) and B.subtilis HS*

The study revived *B.subtilis* strains using LB medium, inoculated into the broth, and kept under shaking at 37°C for 24-48 hours until reaching an optical density of 1 OD, which were then used for further study.



.A B

Fig.3.1 Isolated colonies of *B. subtilis 2049 (T)* as depicted in (A) and *B. subtilis HS* on LB agar plate as depicted in (B).

3.2 Biofilm formation ability of *B.subtilis 2049 (T) and B.subtilis HS*

Researchers have explored the genes involved in B.subtilis' multicellular architectural complex biofilms. They focused on the extracellular polysaccharide matrix that provides rigid support. The wild strain was revived, biofilm formation assays performed, and crystal violet staining was done. The spectrophotometric analysis measured the OD at 595 nm. The mean OD for B. subtilis 2049 (T) and B. subtilis HS was 1.175± 0.184 and 0.876± 0.177, respectively.

3.3 Genomic DNA Isolation.

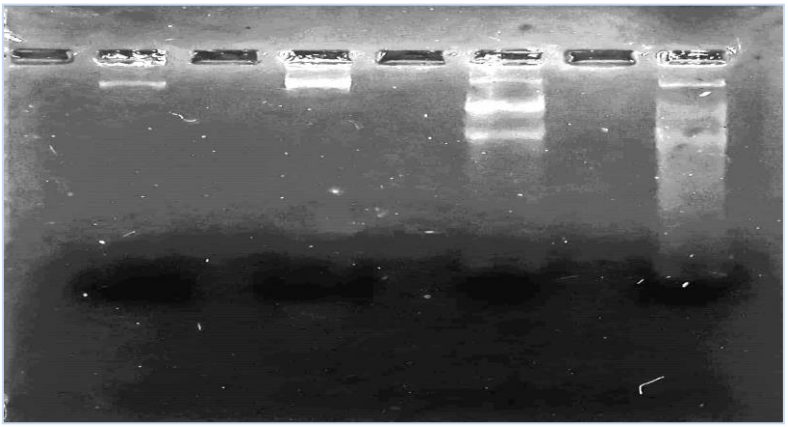


Fig.3.2 Genomic DNA of *B. subtilis 2049 (T)* and *B. subtilis HS* using agarose gel electrophoresis as depicted in lane 2 and lane 4 respectively.

DNA was extracted from a 24-hour-old B.subtilis culture using a manual Isopropanol: chloroform procedure. The sample was eluted with 90% ethanol and displayed in a gel band from two different B.subtilis cultures. The results are depicted in the methods section.

Wavelength (nm)	*B.subtilis 2049 (T)*	*B.subtilis HS*
260 nm	1.72	0.54
280 nm	0.72	0.24

Table.3.1 Optical density of DNA extracted frpm *B.subtilis 2049 (T) and B.subtilis HS* measured at 260nm and 280 nm

The concentration of the isolated DNA sample was determined by multiplying the O.D at 260 nm by 50, resulting in 86 µg/ml for B.subtilis 2049 (T) and 27 µg/ml for B.subtili HS, respectively.

3.4 Detection of *ymcA* gene in *B.subtilis 2049 (T) and B.subtilis HS*

The extracted DNA sample was PCRed using the standard method, using the ymcA gene primer set. The ymcA gene of B. subtilis 2049 (T) and B. subtilis HS was amplified multiple times. The amplification was confirmed through agarose gel electrophoresis with bromophenol blue and UV spectrophotometer. The biofilm forming ability of the ymcA gene was assessed using a microtiter plate assay.

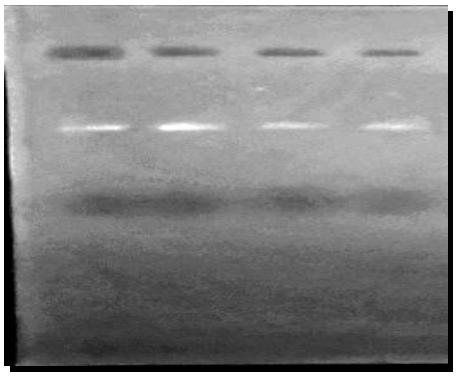


Fig.*3.3 ymcA* gene expression of *B. subtilis 2049 (T)* (Lane 1 & 2) and *B. subtilis HS* (Lane 3 & 4)

3.5 Sequence variation of the *ymcA* gene of *B.subtilis.*

The ymcA protein, a functional protein in B. subtilis, was identified through a NCBI site accession number KI82957.1. The protein encodes 143 amino acids and is involved in biofilm formation. Bioinformatics studies included Multiple Sequence Alignment, phylogenetic analysis, conserved domain identification, and secondary structure prediction for various B. subtilis strains.

3.6 Multiple Sequence Alignment of the *ymcA* gene of *B.subtiis*

The MSA of the *ymcA* protein was obtained through ClustalW tool by using the query sequences in the fasta format.

Multiple sequence alignment (MSA) methods are algorithms used to align evolutionarily related sequences, considering mutations, insertions, deletions, and rearrangements. They can be applied to DNA, RNA, or protein sequences. MSA is widely used in biology, with ClustalW being one of the most cited scientific papers. In silico analyses, such as domain analysis, phylogenetic reconstruction, and motif finding, heavily rely on MSA methods.

The MSA of the ymcA gene of the *B.subtilis* strains *168, RO-NN-1, TU-B-10, and 6051-HGW* is shown below:

```
KIX82957.1      ATGACCCTGTACAGCAAGAAGGACATCGTGCAGCAGGCCAGGAACCTGGCCAAGATGATC
AGG61073.1      ATGACCCTGTACAGCAAGAAGGACATCGTGCAGCAGGCCAGGAACCTGGCCAAGATGATC
YMCA_BACSU      ATGACCCTGTACAGCAAGAAGGACATCGTGCAGCAGGCCAGGAACCTGGCCAAGATGATC
AEP90854.1      ATGACCCTGTACAGCAAGAAGGACATCGTGCAGCAGGCCAGGAACCTGGCCAAGATGATC
AEP86691.1      ATGACCCTGTACAGCAAGAAGGACATCGTGCAGCAGGCCAGGAACCTGGCCAAGATGATC
                ************************************************************

KIX82957.1      AGCGAGACCGAGGAGGTGGACTTCTTCAAGAGGGCCGAGGCCCAGATCAACGAGAACGAC
AGG61073.1      AGCGAGACCGAGGAGGTGGACTTCTTCAAGAGGGCCGAGGCCCAGATCAACGAGAACGAC
YMCA_BACSU      AGCGAGACCGAGGAGGTGGACTTCTTCAAGAGGGCCGAGGCCCAGATCAACGAGAACGAC
AEP90854.1      AGCGAGACCGAGGAGGTGGACTTCTTCAAGAGGGCCGAGGCCCAGATCAACGAGAACGAC
AEP86691.1      AGCGAGACCGAGGAGGTGGACTTCTTCAAGAGGGCCGAGGCCCAGATCAACGAGAACGAC
                ************************************************************

KIX82957.1      AAGGTGAGCACCATCGTGAACCAGATCAAGGCCCTGCAGAAGCAGGCCGTGAACCTGAAG
AGG61073.1      AAGGTGAGCACCATCGTGAACCAGATCAAGGCCCTGCAGAAGCAGGCCGTGAACCTGAAG
YMCA_BACSU      AAGGTGAGCACCATCGTGAACCAGATCAAGGCCCTGCAGAAGCAGGCCGTGAACCTGAAG
AEP90854.1      AAGGTGAGCACCATCGTGAACCAGATCAAGGCCCTGCAGAAGCAGGCCGTGAACCTGAAG
AEP86691.1      AAGGTGAGCACCATCGTGAACCAGATCAAGGCCCTGCAGAAGCAGGCCGTGAACCTGAAG
                ************************************************************

KIX82957.1      CACTACGAGAAGCACGAGGCCCTGAAGCAGGTGGAGGCCAAGATCGACGCCCTGCAGGAG
AGG61073.1      CACTACGAGAAGCACGAGGCCCTGAAGCAGGTGGAGGCCAAGATCGACGCCCTGCAGGAG
YMCA_BACSU      CACTACGAGAAGCACGAGGCCCTGAAGCAGGTGGAGGCCAAGATCGACGCCCTGCAGGAG
AEP90854.1      CACTACGAGAAGCACGAGGCCCTGAAGCAGGTGGAGGCCAAGATCGACGCCCTGCAGGAG
AEP86691.1      CACTACGAGAAGCACGAGGCCCTGAAGCAGGTGGAGGCCAAGATCGACGCCCTGCAGGAG
                ************************************************************

KIX82957.1      GAGCTGGAGGAGATCCCCGTGATCCAGGAGTTCAGGGACAGCCAGATGGAGGTGAACGAC
AGG61073.1      GAGCTGGAGGAGATCCCCGTGATCCAGGAGTTCAGGGACAGCCAGATGGAGGTGAACGAC
YMCA_BACSU      GAGCTGGAGGAGATCCCCGTGATCCAGGAGTTCAGGGACAGCCAGATGGAGGTGAACGAC
AEP90854.1      GAGCTGGAGGAGATCCCCGTGATCCAGGAGTTCAGGGACAGCCAGATGGAGGTGAACGAC
AEP86691.1      GAGCTGGAGGAGATCCCCGTGATCCAGGAGTTCAGGGACAGCCAGATGGAGGTGAACGAC
                ************************************************************

KIX82957.1      CTGCTGCAGCTGGTGGCCCACACCATCAGCAACCAGGTGACCAACGAGATCATCACCAGC
AGG61073.1      CTGCTGCAGCTGGTGGCCCACACCATCAGCAACCAGGTGACCAACGAGATCATCACCAGC
YMCA_BACSU      CTGCTGCAGCTGGTGGCCCACACCATCAGCAACCAGGTGACCAACGAGATCATCACCAGC
AEP90854.1      CTGCTGCAGCTGGTGGCCCACACCATCAGCAACCAGGTGACCAACGAGATCATCACCAGC
AEP86691.1      CTGCTGCAGCTGGTGGCCCACACCATCAGCAACCAGGTGACCAACGAGATCATCACCAGC
                ************************************************************

KIX82957.1      ACCGGCGGCGACCTGCTGAAGGGCGAGACCGGCAGCAAGGTGAAGCACAGCAACAACAGC
AGG61073.1      ACCGGCGGCGACCTGCTGAAGGGCGAGACCGGCAGCAAGGTGAAGCACAGCAACAACAGC
YMCA_BACSU      ACCGGCGGCGACCTGCTGAAGGGCGAGACCGGCAGCAAGGTGAAGCACAGCAACAACAGC
AEP90854.1      ACCGGCGGCGACCTGCTGAAGGGCGAGACCGGCAGCAAGGTGAAGCACAGCAACAACAGC
AEP86691.1      ACCGGCGGCGACCTGCTGAAGGGCGAGACCGGCAGCAAGGTGAAGCACAGCAACAACAGC
                ************************************************************

KIX82957.1      TGCAGCCTG
AGG61073.1      TGCAGCCTG
YMCA_BACSU      TGCAGCCTG
AEP90854.1      TGCAGCCTG
AEP86691.1      TGCAGCCTG
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Fig.3.4 Multiple sequence Alignment of the *ymcA* gene of *Bacillus subtilis* strains *168, RO-NN-1, TU-B-10, and 6051-HGW* using clustalW tool.

MSA results indicate the ymcA gene is conserved in all B.subtilis strains used in this study, as all sequences align perfectly with no gaps.

3.7 Phylogenetic analysis of the *ymcA* gene

The Phylogenetic tree of the related requence was generated usng Blast Tree view (BLAST pairwise alignment) tool using default parameters: Tree view methof of-Fast Minimum Evolution, Maximum Sequence Difference of 0.85.

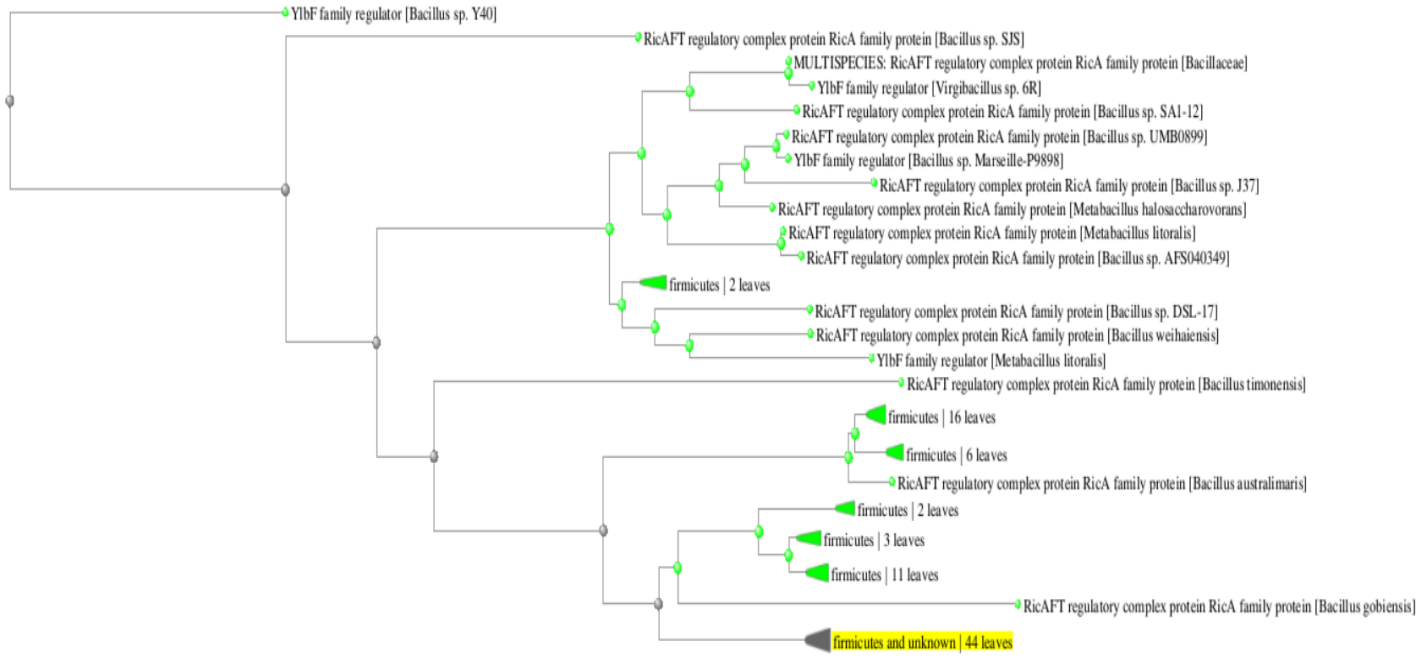

Fig. 3.5 Distant tree result of the *ymcA* gene produced by BLAST tree view tool.

The phylogenetic tree of the *ymcA* gene of the *Bacillus subtilis* strains strains *168, RO-NN-1, TU-B-10, and 6051-HGW.*

Fig. 3.6 Phylogenetic tree of *ymcA* gene of *B.subtilis* strains *168, RO-NN-1, TU-B-10, and 6051-HGW* (https://www.ebi.ac.uk/Tools/phylogeny/simple_phylogeny/)

3.8 Identification of the conserved domain of the *ymcA* protein.

CDD is a protein annotation resource that provides multiple sequence alignment models for ancient domains and full-length proteins. It offers position-specific score matrices (PSSMs) for identifying conserved domains in protein sequences. The conserved domains of the ymcA protein were obtained using standar methods and belong to the RicAFT regulatory complex protein RicA family protein. The ymcA gene is crucial for correct biofilm formation in B.subtilis, and any change in the conserved

domain can destroy the organism's ability to form proper biofilm. The conserved domain is highlighted in red, while less conserved sequences are highlighted in blue.

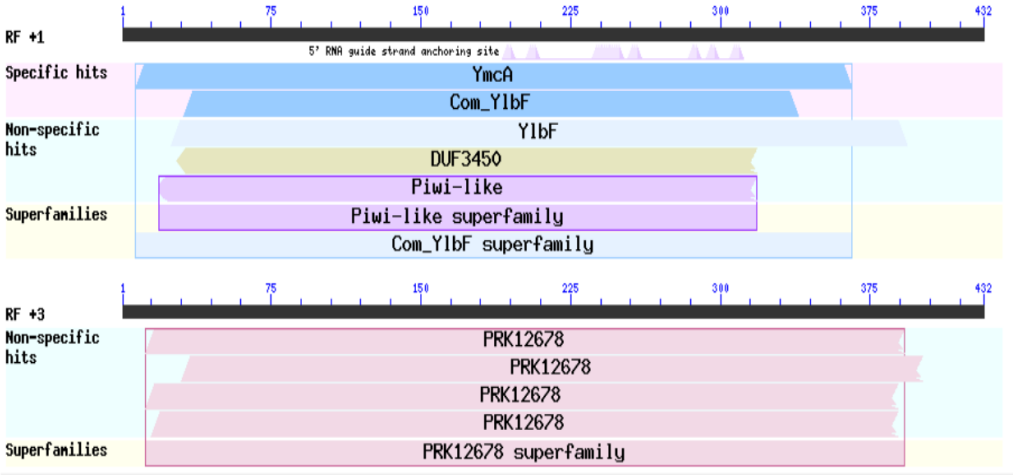

Fig 3.7 Graphical view of the conserved domain of the *ymcA* protein of *B. subtilis.*

3.9 Elucidation of the secondary structure of the *ymcA* protein

The crystal structure of protein ymcA was obtained from the Protein Data Bank (PDB) site, with a secondary structure showing two identical chains and one unique chain. The total structure weight is 34.80 kDa and the atom count is 2179.

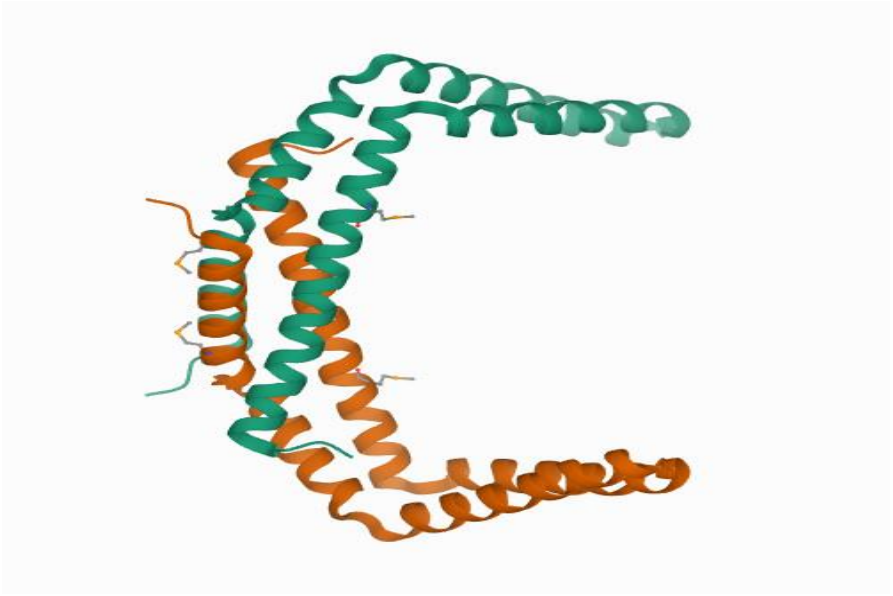

Fig.3.8 Secondary structure of the y*mcA* protein *ymcA* of *B. subtilis* strain.168 obtained from PDB.

The bio-informatics analysis of the ymcA gene in *B. subtilis* strains revealed that it is conserved across all strains and crucial for biofilm formation, suggesting any protein structure changes could lead to incorrect biofilm formation.

4. Conclusion

B. subtilis is a rod-shaped bacterium found in soil and the gastrointestinal tract of humans and ruminants. It forms a protective endospore and can form multicellular structured biofilms, allowing it to drive nutrients from hard-to-reach surfaces. The ymcA gene plays a crucial role in correct biofilm formation. The ymcA gene was detected through PCR, agarose gel electrophoresis, and Microtiter Plate Assay. Bioinformatics studies revealed the importance of the ymcA protein in biofilm formation. Any changes in the protein's structure or gene mutation can result in incorrect biofilm formation, making the organism unfit for survival in external environments.

5. Reference

1. Beauregard, P. B., Chai, Y., Vlamakis, H., Losick, R., & Kolter, R. (2013). Bacillus subtilis biofilm induction by plant polysaccharides. Proceedings of the National Academy of Sciences, 110(17), E1621-E1630.
2. Branda, S. S., Chu, F., Kearns, D. B., Losick, R., & Kolter, R. (2006). A major protein component of the Bacillus subtilis biofilm matrix. Molecular microbiology, 59(4), 1229-1238.
3. Branda, S. S., González-Pastor, J. E., Ben-Yehuda, S., Losick, R., & Kolter, R. (2001). Fruiting body formation by Bacillus subtilis. Proceedings of the National Academy of Sciences, 98(20), 11621-11626.
4. Cairns, L. S., Hobley, L., & Stanley-Wall, N. R. (2014). Biofilm formation by B acillus subtilis: new insights into regulatory strategies and assembly mechanisms. Molecular microbiology, 93(4), 587-598.
5. Carabetta, V. J., Tanner, A. W., Greco, T. M., Defrancesco, M., Cristea, I. M., & Dubnau, D. (2013). A complex of YlbF, YmcA and YaaT regulates sporulation, competence and biofilm formation by accelerating the phosphorylation of Spo0A. Molecular microbiology, 88(2), 283-300.
6. Chu, F., Kearns, D. B., McLoon, A., Chai, Y., Kolter, R., & Losick, R. (2008). A novel regulatory protein governing biofilm formation in Bacillus subtilis. Molecular microbiology, 68(5), 1117-1127.
7. Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., & Lappin-Scott, H. M. (1995). Microbial biofilms. Annual review of microbiology, 49(1), 711-745.
8. Dietmann, S., Holm, L. Identification of homology in protein structure classification. Nat Struct Mol Biol 8, 953–957 (2001).
9. Donlan, R. M. (2002). Biofilms: microbial life on surfaces. Emerging infectious diseases, 8(9), 881.
10. Drouin, R., Rodriguez, H., Gao, S. W., Gebreyes, Z., O'Connor, T. R., Holmquist, G. P., & Akman, S. A. (1996). Cupric ion/ascorbate/hydrogen peroxide-induced DNA damage: DNA-bound copper ion primarily induces base modifications. Free Radical Biology and Medicine, 21(3), 261-273.
11. Earl, A. M., Losick, R., & Kolter, R. (2008). Ecology and genomics of Bacillus subtilis. Trends in microbiology, 16(6), 269-275.
12. Fawcett, P., Eichenberger, P., Losick, R., and Youngman, P. (2000) The transcriptional profile of early to middle sporulation in Bacillus subtilis. Proc Natl Acad Sciences USA 97: 8063–8068.
13. Fujita, M., González-Pastor, J. E., & Losick, R. (2005). High-and low-threshold genes in the Spo0A regulon of Bacillus subtilis. Journal of bacteriology, 187(4), 1357-1368.
14. Grubbs, K. J., Bleich, R. M., Santa Maria, K. C., Allen, S. E., Farag, S., Team, A., ... & Bowers, A. A. (2017). Large-scale bioinformatics analysis of Bacillus genomes uncovers conserved roles of natural products in bacterial physiology. MSystems, 2(6).

15. Hamon, M. A., & Lazazzera, B. A. (2001). The sporulation transcription factor Spo0A is required for biofilm development in Bacillus subtilis. Molecular microbiology, 42(5), 1199-1209.
16. Hamon, M. A., Stanley, N. R., Britton, R. A., Grossman, A. D., & Lazazzera, B. A. (2004). Identification of AbrB-regulated genes involved in biofilm formation by Bacillus subtilis. Molecular microbiology, 52(3), 847-860.
17. Harwood, C. R., & Cutting, S. M. (1990). Molecular biological methods for Bacillus. Wiley.
18. Kearns, D. B., & Losick, R. (2003). Swarming motility in undomesticated Bacillus subtilis. Molecular microbiology, 49(3), 581-590.
19. Kearns, D. B., Chu, F., Branda, S. S., Kolter, R., & Losick, R. (2005). A master regulator for biofilm formation by Bacillus subtilis. Molecular microbiology, 55(3), 739-749.
20. Kobayashi, K. (2008). SlrR/SlrA controls the initiation of biofilm formation in Bacillus subtilis. Molecular microbiology, 69(6), 1399-1410.
21. Lemon, K. P., Earl, A. M., Vlamakis, H. C., Aguilar, C., & Kolter, R. (2008). Biofilm development with an emphasis on Bacillus subtilis. In Bacterial biofilms (pp. 1-16). Springer, Berlin, Heidelberg.
22. López, D., Fischbach, M. A., Chu, F., Losick, R., & Kolter, R. (2009). Structurally diverse natural products that cause potassium leakage trigger multicellularity in Bacillus subtilis. Proceedings of the National Academy of Sciences, 106(1), 280-285.
23. Marchler-Bauer, A., Bo, Y., Han, L., He, J., Lanczycki, C. J., Lu, S., ... & Gwadz, M. (2017). CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. Nucleic acids research, 45(D1), D200-D203.
24. Mielich-Süss, B., & Lopez, D. (2015). Molecular mechanisms involved in B acillus subtilis biofilm formation. Environmental microbiology, 17(3), 555-565.
25. olle, V., Fujita, M., Jensen, S. T., Eichenberger, P., González-Pastor, J. E., Liu, J. S., & Losick, R. (2003). The Spo0A regulon of Bacillus subtilis. Molecular microbiology, 50(5), 1683-1701.
26. Murray, E. J., Strauch, M. A., & Stanley-Wall, N. R. (2009). σX is involved in controlling Bacillus subtilis biofilm architecture through the AbrB homologue Abh. Journal of bacteriology, 191(22), 6822-6832
27. Pearce, P., Song, B., Skinner, D. J., Mok, R., Hartmann, R., Singh, P. K., ... & Dunkel, J. (2019). Flow-induced symmetry breaking in growing bacterial biofilms. Physical Review Letters, 123(25), 258101.
28. Sakthivel, S., & Habeeb, S. K. M. (2015). NNvPDB: neural network based protein secondary structure prediction with PDB validation. Bioinformation, 11(8), 416.
29. Sambrook, J., & Russell, D. (2001). Molecular cloning: a laboratory manual (3-Volume Set) 3 Lab edition.
30. Stanley, N. R., & Lazazzera, B. A. (2005). Defining the genetic differences between wild and domestic strains of Bacillus subtilis that affect poly-γ-DL-glutamic acid production and biofilm formation. Molecular microbiology, 57(4), 1143-1158.
31. Steinberg, N., Keren-Paz, A., Hou, Q., Doron, S., Yanuka-Golub, K., Olender, T., ... & Romero, D. (2020). The extracellular matrix protein TasA is a developmental cue that maintains a motile subpopulation within Bacillus subtilis biofilms. Science Signaling, 13(632).
32. Ye, J., McGinnis, S., & Madden, T. L. (2006). BLAST: improvements for better sequence analysis. Nucleic acids research, 34(suppl_2), W6-W9.