Molecular insights of endospore like refractile bodies of *Rhodobacter johrii* JA192

Thesis submitted to the University of Hyderabad for the award of

Doctor of Philosophy

By

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This is to certify that **Mr. ASHIF ALI** has carried out the research work embodied in the present thesis under the supervision and guidance of **Prof. Ch. Venkata Ramana** for a full period prescribed under the Ph.D. ordinances of this University. We recommend his thesis entitled **"Molecular insights of endospore like refractile bodies of *Rhodobacter johrii* JA192"** for submission for the degree of Doctor of Philosophy of the University.

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I **Ashif Ali**, hereby declare that the work embodied in this thesis entitled "***Molecular insights of endospore like refractile bodies of Rhodobacter johrii* JA192"** has been carried out by me under the supervision of **Prof. Ch. Venkata Ramana**, Department of Plant Sciences, School of Life Sciences. The work presented in this thesis is a bonafide research work and has not been submitted for any degree or diploma in any other University or Institute. A report on plagiarism statistics from the University Librarian is enclosed.

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A. Published in the following journal :

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Further the student has passed the following courses toward the fulfillment of the coursework requirement for Ph.D. degree.

S.No.	Course code	Name	Credits	Pass/Fail
1.	PL801	Seminar 1	1	Pass
2.	PL802	Research ethics and management	2	Pass
3.	PL803	Biostatistics	2	Pass
4.	PL804	Analytical Techniques	3	Pass
5.	PL805	Lab Work	4	Pass

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Ashif Ali

DEDICATED TO MY BELOVED PARENTS AND WELL-WISHERS

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2.3 Bioinformatical (*in silico*) methodology

2.4 Protein characterization

List of Abbreviations

°C	Degree Celsius
µg	Microgram
µl	Micro litre
µM	Micro molar
Å	Angstrom
ANI	Average nucleotide identity
BLAST	Basic Local Alignment Search Tool
BLASTn	Nucleotide Basic Local Alignment Search Tool
BLASTp	Protein Basic Local Alignment Search Tool
bp	Base pair
BPGA	Bacterial pan genome analysis
BSA	Bovine serum albumin
CDS	Coding sequences
DAPI	4,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamino tetra acetic acid
EMBL	European molecular biology laboratory
g	Gram
$g.l^{-1}$	Gram per liter
h	Hour
IPTG	Isopropyl-β-D-1 thiogalactopyranoside
K	Kelvin
Kb	Kilo base
kDa	Kilo Dalton
L	litre
lux	luminous flux per unit area
M	Molar
mg	Milli gram
min	Minute
ml	Milli litre
mM	Millimolar
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
nm	Nano metre
nt	Nucleotide
OD	Optical density

PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDB	Protein Data bank
PMSF	Phenylmethylsulfonyl fluoride
RAST	Rapid annotations using subsystems technology
Rba.	*Rhodobacter*
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT	Room temperature
s	Seconds
SDS – PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	Second
SEM	Scanning electron microscope
TBE	Tris borate EDTA-buffer
TEM	Transmission electron microscope
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume

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INTRODUCTION

1. Introduction

1.1 Bacterial survival adaptations

Microorganisms survive in natural habitats that are highly dynamic in physicochemical stressful environment like extreme temperatures (high temperatures [hot springs, thermal vent] and low temperature [Antarctica regions]), lack of nutrients, high salinity, toxic chemicals, unfavorable moisture content, alkaline and acidic conditions with limited resources for viability. The major question arise is "How do bacteria survive?" Bacteria has came up with several strategies which are developed through adaptive mechanisms. The adaptation mechanisms developed by bacteria are based on the molecular and cellular bases (Bleuven and Landry, 2016). The adaptive strategies developed as a response of bacteria for survival are as follows:

- Biofilm formation
- Viable But Non-culturable (VBNC) state
- Cyst formation
- Spore formation
  - (i) Exospores
  - (ii) Endospores

1.1.1 Biofilm formation

When bacterial communities surrounded by an extracellular matrix (ECM) are attached at surface is called biofilm (Monds and Toole, 2009) (Fig. 1.1A). In mixed or multispecies biofilm the microorganisms adhere to solid surface and makes a complex of multispecies embedded in extracellular polymeric substance (EPS) generated by microorganisms (Hall-Stoodley *et al*., 2004). Extracellular polymeric substance is the formation of surface polysaccharides which involves compounds made of carbohydrate polymers (Flemming *et al*., 2016). In biofilm

formation same genes are responsible which participates in chemotaxis, creating aggregation on surface. In biofilm formation, bacteria also become resistant and tolerant to antibiotics clinically (Wu *et al.,* 2015).

1.1.2 Viable But Non-culturable (VBNC) state

The cells at Viable But Non-culturable (VBNC) are non-dividing but metabolically active (Fig. 1.1B), viable and resuscitated when favorable conditions arrives as seen in many members of various bacterial phyla (Nowakowska and Oliver, 2013). It was reported in *Escherichia coli, Vibrio* spp. (*Vibrio cholerae, Vibrio vulnificus* and a few other species) (Xu *et al*., 1982), *Campylobacter jejuni*, *Salmonella* spp., *Helicobacter pylori*, *Shigella* spp., *Enterobacter* Spp., *Listeria monocytogenes*, *Pseudomonas* spp., *Staphylococcus aureus* and *Mycobacterium tuberculosis*. Many of the bacterial pathogens show this survival strategy of dormancy.

1.1.3 Cyst formation

A thick walled dormant structures having capsular slime are cysts (Fig. 1.1C), which are lesser resistant comparably to endospore. In bacteria, cysts are formed by *Azotobacter* spp., *Rhodospirillium centenum* (Berleman and Bauer, 2004), *Myxobacteria, Clamydobacteria, Azotobacter* spp., methane users *Methylocystis parvus*, all *Methylococcus* and *Methylomonas* group and all types of *Methylobacter* (*M. chroococcum*, *M. capsulatus*, *M. bovis*, *M. vinelandii*) (Whittenbury *et al*., 1970). Recently in *Serratia ureilytica* isolated an extreme thermophilic conditions is also shown to form cyst (Filipidou *et al*., 2019). Cysts are resistant to ultraviolet radiation, gamma radiation, desiccation and sonic treatment however lesser to heat (Socolofsky and Wyss, 1962).

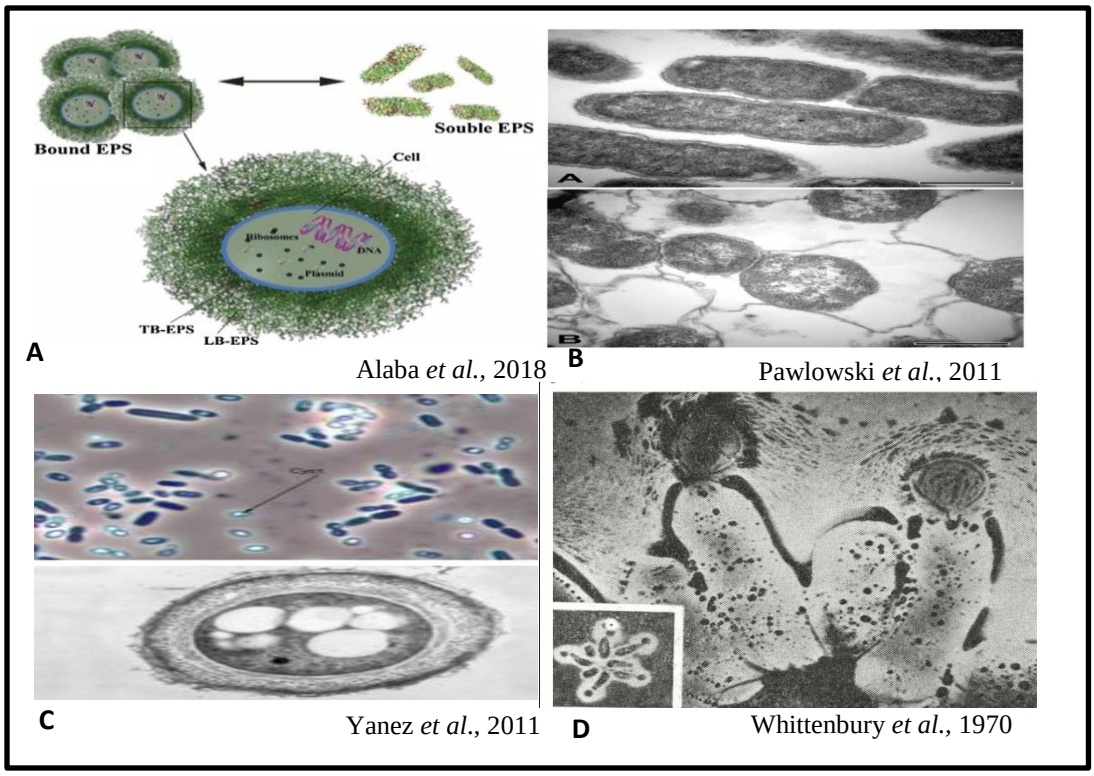


Figure 1.1 Different dormant structures in bacteria A. EPS and Biofilm formation B. VBNC State C. Cyst, heterocyst D. Exospores

1.1.4 Spore formation

A highly resistant, tough and dormant body made to face extreme conditions of disinfectants, intense heat and U.V radiation. Endospores first discovered by Cohn (Drews, 2000) in the firmicute *Bacillus subtilis,* then by Koch (Drews, 2000) in pathogen *Bacillus anthracis.* Spores are the most durable type of dormant bodies on earth which can remain viable for millions of year. Two types of spores are produced by bacteria; exospores and endospores.

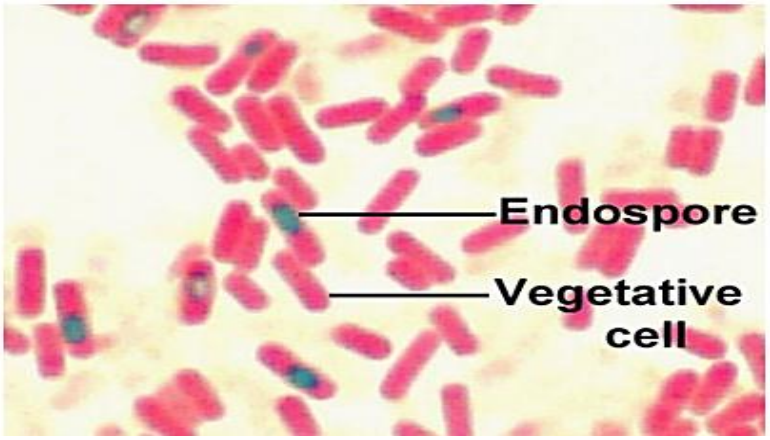
(i) Exospores

Exospores are structures formed outside the vegetative cell by budding at one pole of cell and come out as a bud (Fig. 1.1D). Exospores are produced by methane-oxidizing bacteria from the genus *Methylosinus* belongs to Proteobacteria like *M. trichosporium* (Reed *et al.,* 1980; Reed and

Dugan, 1979). Genus *Streptomyces* and *Actinomyces* belonging to the *Actinobacteria* groups also produces exospores (Ryding *et al*., 1998)

(ii) Endospores

When tough, dormant structures are formed within the vegetative cell it is called endospore (Fig. 1.2). Endospores are formed by firmicute members of *Bacillus* spp. and *Clostridium* spp. (Hutchinson *et al.*, 2016; Piggot and Hilbert, 2004) within the mother cell. The most resistant dormant bodies which is heat and U.V irradiation resistant.



Chamberlain *et al.*, 2009

Figure 1.2 Stained endospores of *Bacillus subtilis*.

1.2 Bacterial sporulation

Formation of endospores has been extensively studied as compared to the other survival strategies listed above. Endospores are developed under unfavorable conditions which later germinate when favorable conditions resume. Over the recent past, many cases have been reported which showed the presence of endospores-like bodies or dormant bodies in bacteria from taxa other than Firmicutes members like *C. burnetii* (McCaul and Williams, 1981), *S. marcescens* (Ajithkumar *et al*., 2003), *Myxococcus xanthus* (Kroos, 2007), few *Mycobacterium* spp. (Singh *et al.*, 2010; Ghosh *et al*., 2009; Lamont *et al*.,2012), *Rba. johrii* (Girija *et al.*, 2010),

R. palustris (Venkidusamy and Megharaj, 2016), *Serratia ureilytica* Lr5/4 (Filippidou *et al.*, 2019).

1.3 Firmicutes

The process of sporogenesis has been greatly explored over the years and presently we have deep knowledge on the molecular machinery of sporogenesis of a few Firmicutes wherein *Bacillus subtilis* has been the favorite model organism (Hutchinson *et al*., 2016; Piggot and Hilbert, 2004). *Bacillus subtilis* (similarly in *Clostridium* spp.) undergo sporulation which is a developmental program during environmental stress conditions. Sporulation starts with the asymmetrical cell division along with DNA replication and the two copies of genome distributed in both compartments. The smaller compartment is the forespore (Prespore) which develops into the mature spore surrounded by multilayers coat shell composed of many proteins that protects the genome of bacteria during harsh conditions and when the environment is favorable spore germinates and enters into vegetative cycle.

The series of development stages which leads to spore are as follows (Errington, 2003; Higgins and Dworkins, 2012; Setlow, 2007) (Fig. 1.3).

1. Vegetative state
2. Stage starvation-State- activation of Spo0A
3. Stage I-II- Onset of sporulation.
4. Stage II-III- Sporulation and engulfment.
5. Stage III-IV- Completion of engulfment and cortex synthesis.
6. Stage V-VI- Coat synthesis and spore maturation
7. Stage VII- Mother cell lysis.
8. Spore germination and outgrowth.

Vegetative state

In this binary fission occur in the bacteria leads to cell growth this occurs on the availability of nutrients. Cell division cycle progress along with the chromosomal replication, the sister cells are separated by cleavage of cell wall.

Stage starvation - State-activation of Spo0A

When bacteria are facing the stress condition mostly nutrient deficiency act as a molecular signal. This signal is transferred by sporulation-specific two component switches and multicomponent phosphorelay. Signal activates auto phosphorylation of histidine sensor kinases (KinA-KinE) leading to transfer of phosphoryl group through 30 extended phosphorelay to response regulator Spo0F and Spo0B a phosphotransferase which finally phosphorylates and activates master regulator transcription factor of sporulation Spo0A.The phosphorylated Spo0A (Spo0A~P) levels are increased and it binds to 0A boxes upstream of 120 genes are regulated. This stage requires the sigma factors σ^A and σ^H.

Stage I-II - Onset of sporulation

In this stage axial filamentation occurs and here cell has two chromosomes for mother cell and forespore. A condensed form of duplicated chromosomes is called axial filament spread between the two poles of cells. RacA protein helps in anchoring two chromosomes to poles. Spo0A~P along with the sigma factor σ^H activates onset of sporulation by inducing the transcription of genes promoting asymmetric cell division and chromosome elongation. *SpoIIA* operon expresses sporulation sigma factor σ^F and its regulators which are important for the forespore development through the cell division which is unequal by forming asymmetric septum, this happens due to the formation of two polar Z-rings with the help of FtsZ and SpoIIE

protein. SpoIIE plays two major roles first it interacts with FtsZ and the central Z-ring formation is shifted towards poles secondly it activates the pro-σ^F.

Stage II-III - Sporulation and engulfment.

Here asymmetric cell division is completed through the sigma factor σ^E and σ^F present in the mother cell and forespore respectively. Due to polar division, the 30% of one of the two chromosomes is present into the forespore and the remaining chromosome in the mother cell due to this genetic asymmetry, there is a compartment specific gene expression. The remaining 70% of the chromosome in mother cell is then shifted into the forespore with the help of DNA translocase SpoIIIE. When the σ^F activates in the forespore and σ^E in the mother cell which initiates the expression of first set of sporulation specific genes involving those of engulfment process.

Stage III-IV- Completion of engulfment and cortex synthesis.

The forespore is engulfed completely by engulfing membranes and separated by mother cell membrane. Engulfment completion releases forespore into cytoplasm of the mother cell. The forespore is now independent separate body with its own double membrane within mother cell. In Stage IV a thick layer of peptidoglycan is formed between two forespore membranes which forms the spore cortex and building of a thick proteinaceous coat surrounding the forespore. When σ^E in mother cell and σ^G in the forespore expressed these activate σ^K in the mother cell. The compartmentalized gene expression consists of regulatory cascades both in forespore and mother cell. The activation of σ^G and σ^K produces regulators GerE and SpoVT which fine tunes sporulation–specific genes leads to feed-forward loops. A newly discovered transcription modulating protein YlyA is a RNA polymerase-binding protein activates σ^G guided gene

transcription which involves in spore germination and further expression with σ^G and SpoVT. In this stage proteins involve in spore coat and cortex layers are highly synthesize.

Stage V-VI- Coat synthesis and spore maturation

The transcription of the proteins which are involved in the protection of DNA occurs in forespore. Mother cell produces large amount of dipicolinic acid which is transferred into the forespore. Coat proteins and cortex are synthesized in both the compartments which develop a cortex layer composed of thick peptidoglycan and several coat layers around the spore as a protective layer.

Stage VII- Mother Cell lysis

Detachment process occurs in this stage in which forespore is proceeds towards the middle of the cell from the pole, then after some time mother cell cytoplasmic membrane ruptures and succeeds to mother cell wall lysis with the help of CwlH and CwlC cell wall amidase. A released spore is an independent dormant body which is highly resistant to acids, heat, desiccation and other environmental stresses. CwlH and CwlC produces N-acetyl-muramoyl-L-alanine amidase cleaving the peptidoglycans of mother cell, these two hydrolases are transcribed by σ^K also control late gene expression of mother cell specific.

Spore germination and outgrowth

Spores respond to the surroundings on being in dormancy and metabolically inactive this results in spore germination at favorable condition. When the spore senses the trigger molecules like nutrients and peptidoglycan fragments it germinates by lysing the protective cortex and coat layers. Nutrient germinant binds to the germination (Ger) receptor of inner membrane of spore initiates the germination process. Also, the shredded peptidoglycan acts as a signal for germination sensed by Ser/Thr Kinase PrkC. The signal is passed to spore which is committed to

germination liberates the Ca^{+}-DPA out of spore core and water remains rehydrated into core. The degradation of cortex followed by swelling of the core. Finally, the metabolic activity starts for outgrowth of a vegetative cell.

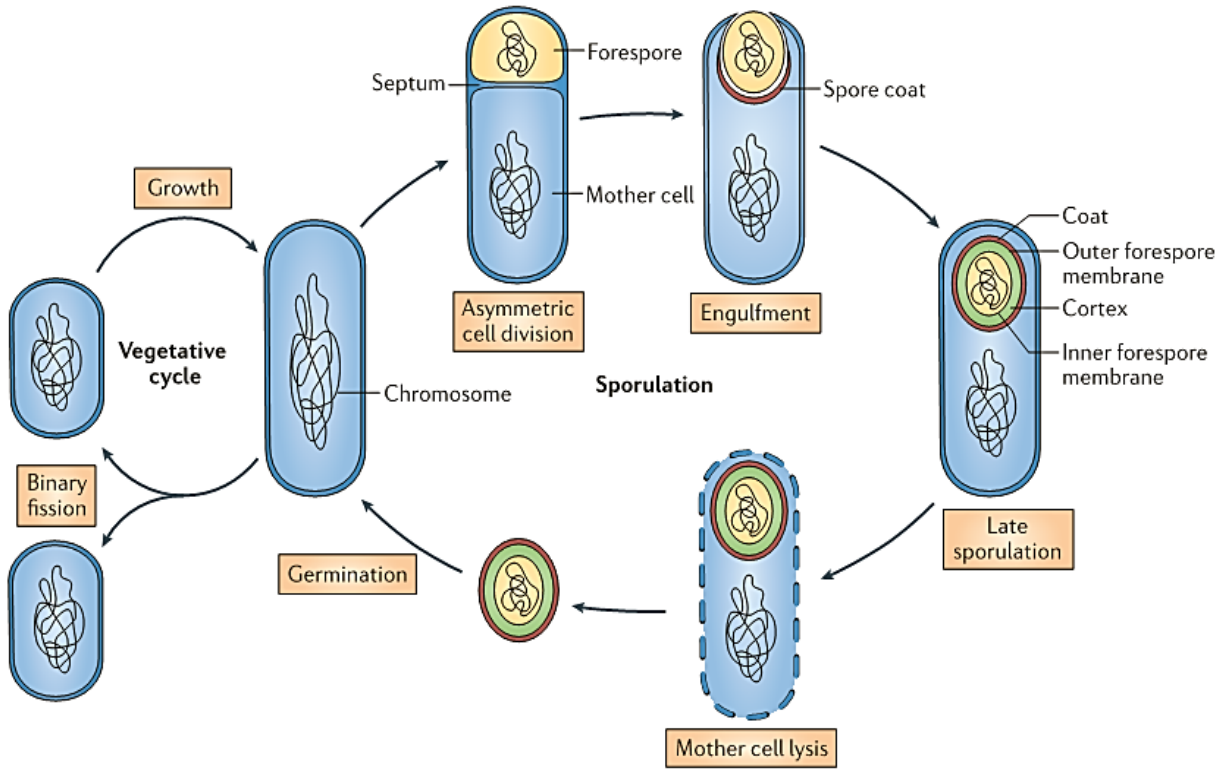


Figure 1.3 Developmental cycles of Firmicute sporulation and germination. A vegetative cell in a stress signal starts dividing asymmetrically into two compartments, mother cell and forespore separates by septum later mother cell engulf the forespore then cortex and coat is formed around the spore including other material makes spore mature finally mother cell ruptures to free the spore, spores sense the favorable conditions for germination and enters into vegetative state. (Image adopted from McKenney *et al.*, 2013).

1.4 Firmicute endospore structure

The mature endospore is a complex structure consist of many layers (Fig. 1.4). Central core contains DNA enclosed within a spore inner membrane. Which is enclosed within the two layer made up of peptidoglycan known to be cortex. Three-layered coat composed of proteins

and peptides encloses the cortex. At last the coat is surrounded within a loose structure composed of glycoprotein called exosporium. These outer layers enclose a compartment with the gap.

The mother cell makes and install all the parts after the inner layer coat proteins, exosporium (Henriques and Moran, 2007) (Fig. 1.5). The spore becomes the resistant by desiccation and accretion of small acid soluble proteins (SASP) there role is to safeguard the DNA of spore and during germination as a source of amino acid. Resistance is provided by the dipicolinic acid incorporation in the cortex and helps in germination (Magge *et al*., 2008).

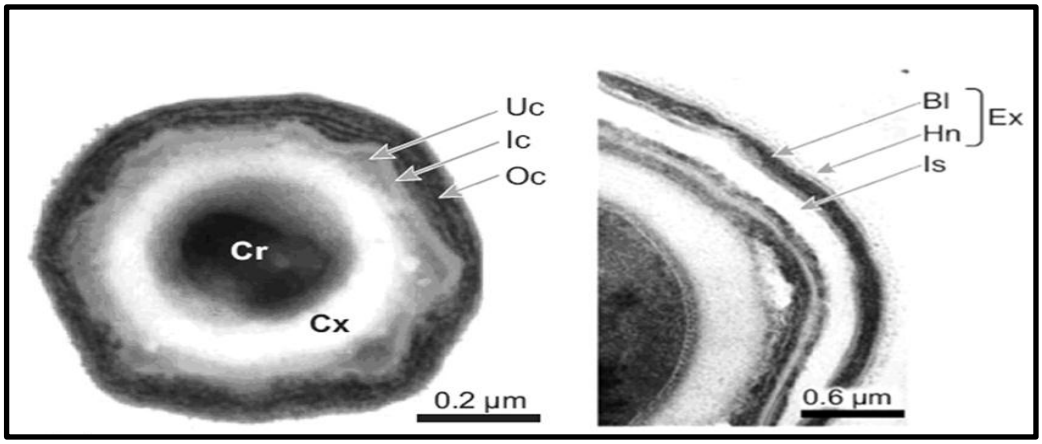
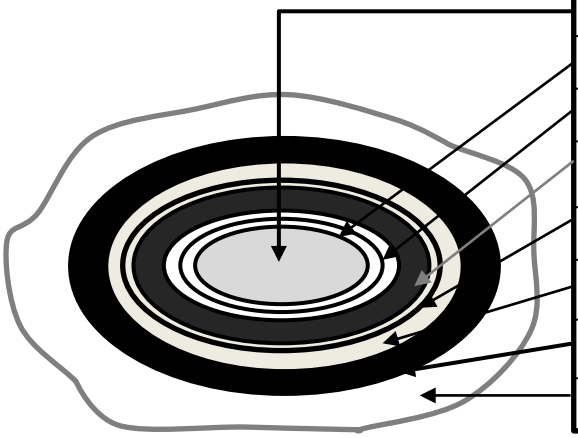


Figure 1.4 Spore structure: Oc: outer coat, Ic: inner coat, Uc: undercoat, Cr: core, Cx: cortex, Ex: exosporium, Is: inter space, Hn: glycoprotein nap, Bl: basal layer, Adopted through (Waller *et al.*, 2004).



Structure	Components
Core	DNA, RNA, Proteins, SASPs, DPA, $Ca2_{+}$,
Inner membrane	Lipid/protein
Germ cell wall	Peptidoglycan
Cortex	Modified Peptidoglycan
Outer membrane	Lipid/protein
Inner spore coat	Proteins
Outer spore coat	Proteins
Exosporium	Proteins

Figure 1.5 Sketch of a mature spore structure along with its components.

1.5 Sporulation in Actinobacteria

The Gram positive Actinobacteria such as *Streptomyces*, *Actinomyces* and *Micromonospora* differentiates into the dormant exospores. The model system for exosporulation study is *Streptomyces*, in its life cycle it forms hypha, these hyphae under stress conditions divides into the compartments where spores are develops, matures, disperse and germinates (Fig. 1.6).

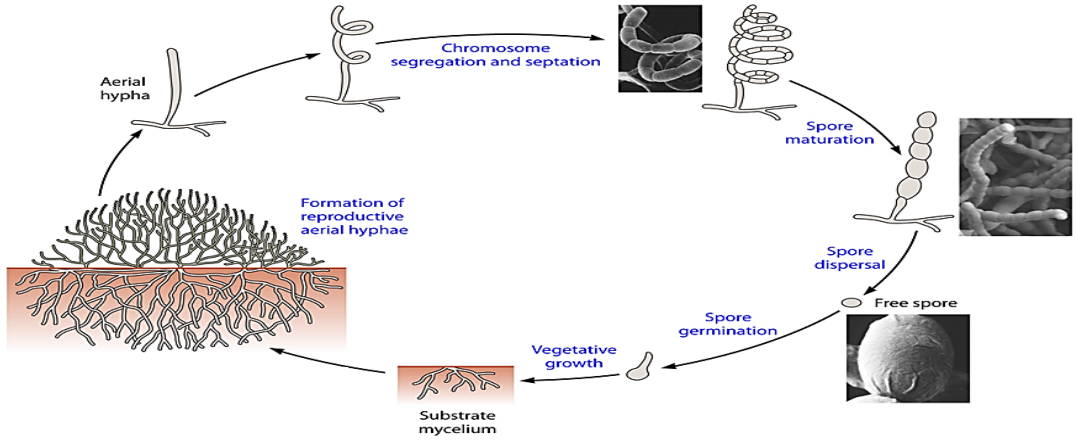


Figure 1.6 Life cycles of Actinomycetes (Adopted from Barka *et al.,* 2016)

Streptomyces normally grows the branch hyphae and nutrient depletion conditions act as a signal for the differentiation leading to the exospores. Activation of sporulation specific regulon occurs through the release of repressor BldD-c-di-GMP complex (Bush *et al.,* 2015; Tschowri *et al.,* 2014). The aerial hyphae now cover with arodlet layer of amyloid fibrils (Wildermuth *et al.,* 1971) which is composed of rodlin and chaplin proteins (Claessen *et al.,* 2004). This sporogenic hyphae is dissected through the septa along with the distribution of chromosome into the prespore compartment (Ausmees *et al.,* 2007; Ditkowski *et al*., 2013; Jakimowicz and van Wezel, 2012;). SsgA-like family proteins (SALP) regulate this process which is present only in Actinomycetes (Sigle *et al.,* 2015). SsgA is an important sporulation protein of *S. coelicolor* also the SsgB that regulates the FtsZ rings assembly in septation sites (Keijser *et al.,* 2003; Van Wezel *et al.,* 2000; Willemse *et al*., 2011; Sevcikova and Kormanec,

2003). SsgD protein has function in developing spore wall (Noens *et al.*, 2005, 2007). Therefore the aerial hyphae turn into the mature spores. During maturation simultaneously DNA condensation and formation of gray pigment occurs in the *S. coelicolor* spore (Kelemen *et al*., 1998). A thin rodlet layer of fibrous sheath is formed surrounding the wall of spore (Claessen *et al*., 2002, 2003). Cell wall hydrolases SsgE and SsgF separate the spores which helps further in the dispersal (Traag and van Wezel, 2008; Haiser *et al*., 2009) (Fig. 1.7).

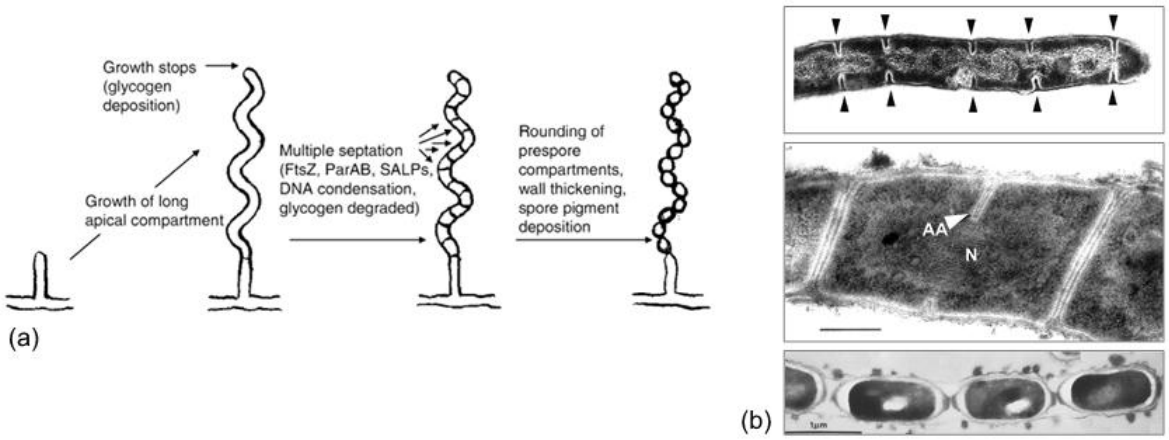


Figure 1.7 (a) Diagrammatic presentation of sporulation in the actinobacteria *S. coelicolor* (Adopted through Chater and Chandra, 2006) (b) Sporulation of *S. coelicolor,* sporulation septa grows to separate the spore. N: nucleoid; AA: Annulus in growing, (Adopted through Hardisson and Manzanal, 1976).

1.6 Other members

Many previous reports has shown dormancy in the bacteria not belonging to the firmicute groups, most of these bacteria are isolated from harsh adverse environment conditions, therefore these bacteria are habitats of that environment which is sometime unfavorable for survival, these bacteria might have develop survival strategy leading to formation of resistant, tough and dormant structures. These dormant bodies have some similarity with the spores of firmicutes but their structures are different at some points. We will discuss the dormancy reported till date other than Firmicute group.

a) ***Coxiella burnetii*** is an intracellular bacterium an agent of Q fever, which produces endospores (McCaul and Williams, 1981) which can be seen at pole of the cell (Fig. 1.8A). *Coxiella burnetii* is reported to be resistant at high temperature 74°C, chemical and physical agents.

b) ***Serratia marcescens*** subsp. sakuensis is gram negative bacterium isolated from the waste-water treatment tank. Under TEM analysis the endospore structure was observed (Fig. 1.8B) having similarity to that of *B. megaterium* endospore (Ajithkumar *et al*., 2003). This bacterium can survive at high temperature of 75°C and high salt concentrations similar to spores.

c) ***Myxococcus xanthus*** develops the fruiting bodies which differentiate into spherical myxospores which are found to be heat resistant (Fig. 1.8C). This type of sporulation is called fruiting body sporulation which is starvation–dependent sporulation (Licking *et al.*, 2000).

d) ***Mycobacterium*** spp. forming dormant endospore like structure was reported by two different groups. *Mycobacterium avium* subsp. *paratuberculosis* old cultures showed dormant spore structures (Fig. 1.8D) they are resistant to lysozyme, proteinase K and heat (Lamont *et al.,* 2012). *Mycobacterium marinum* and *Mycobacterium bovis* also produce endospores (Fig.1.8D) (Singh *et al.,* 2010; Ghosh *et al.,* 2009) and appeared to be refractile bodies under phase contrast microscope (Fig. 1.8D). Therefore it is predicted due to formation of dormant bodies in the *Mycobacterium* spp. persists for long time in the host and resist for drugs.

e) ***Rhodobacter johrii*** strain JA192 produce refractile endospore like structures which stained with malachite green (Fig. 1.9A). Spores are produced from the old culture or stressed cultures (Girija *et al.*, 2010)

f) ***Rhodopseudomonas palustris*** strain RP2 colonies when kept for 4-5 weeks long period incubation at an anoxic chemosynthetic environment produces spores (Fig. 1.9B) (Venkidusamy and Megharaj, 2016).

g) ***Serratia ureilytica*** Lr5/4 isolated from polyextreme environment geothermal spring its temperature exceeds ~54°C has shown refractile cyst like bodies (Fig. 1.9C) in old culture grown in solid media. The Lr5/4 culture has been withstanding a dry heat shock of 75°C (Filippidou *et al.*, 2019).

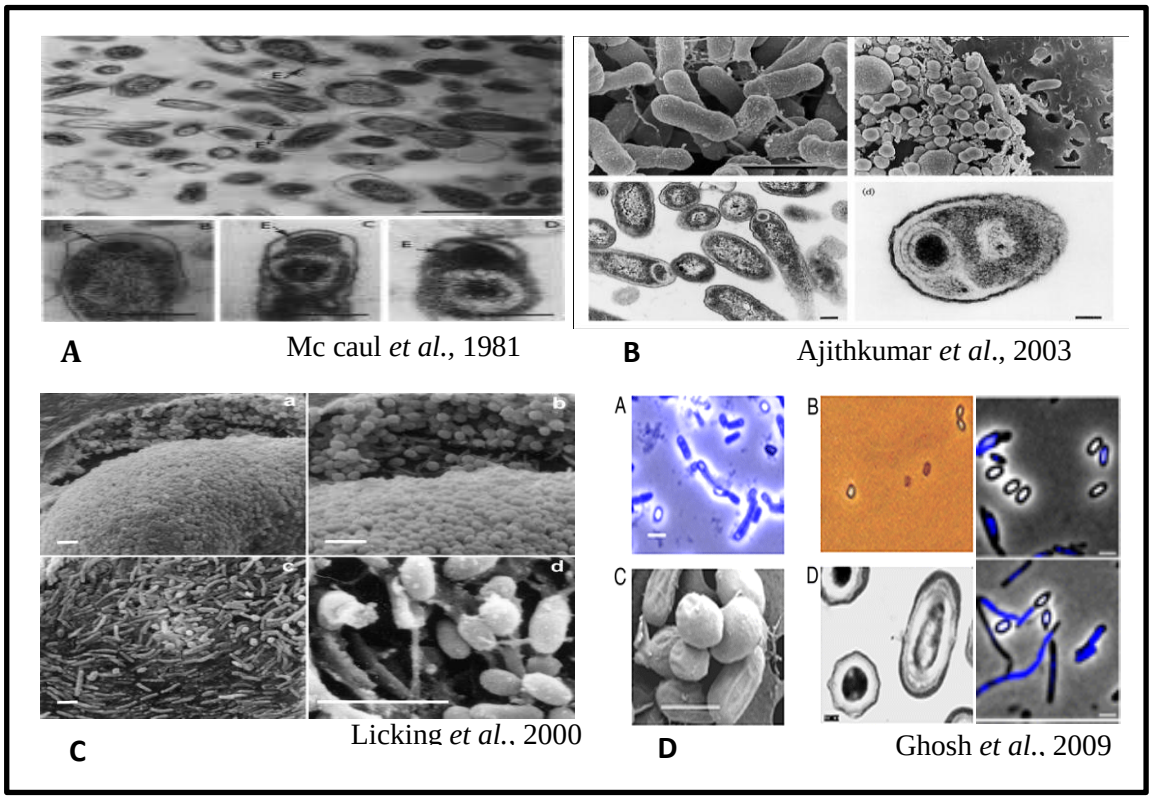


Figure 1.8 Dormant bodies of non firmicute bacteria (A) *Coxiella burnetii* (B) *Serratia marcescens* subsp. *sakuensis* (C) *Myxococcus xanthus* (D) *Mycobacterium bovis, M. marinum.*

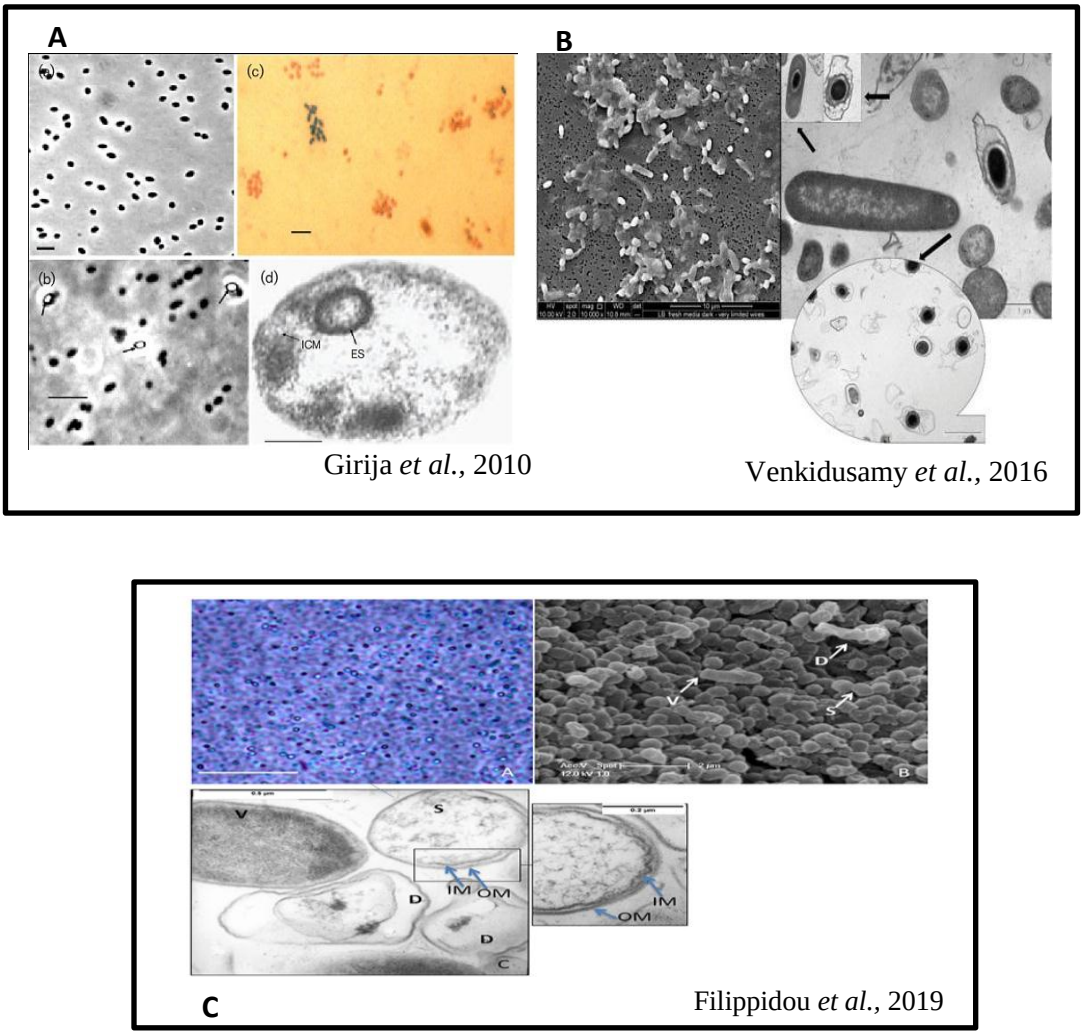


Figure 1.9 Dormancy of bacterial cells (A) *Rhodobacter johrii* JA192 (B) *Rhodopseudomonas palustris* RP2 (C) *Serratia ureilytica* Lr5/4

1.7 Structure of bacterial cell wall peptidoglycan

Bacterial cell wall is a cross linked polymer which surrounds the bacteria made up of peptidoglycan polymer, a repeat of N-Acetylglucosamine and N-Acetylmuramic acid (NAG and NAM) attached through β 1-4-glycosidic bond by transglycosylase. NAM has peptides stem out from it these peptides are di, tri and tetrapeptides. The parallel neighbor strands are cross-linked by DD-trans-peptidase (Fig. 1.10) (Rogers *et al.*, 1981).

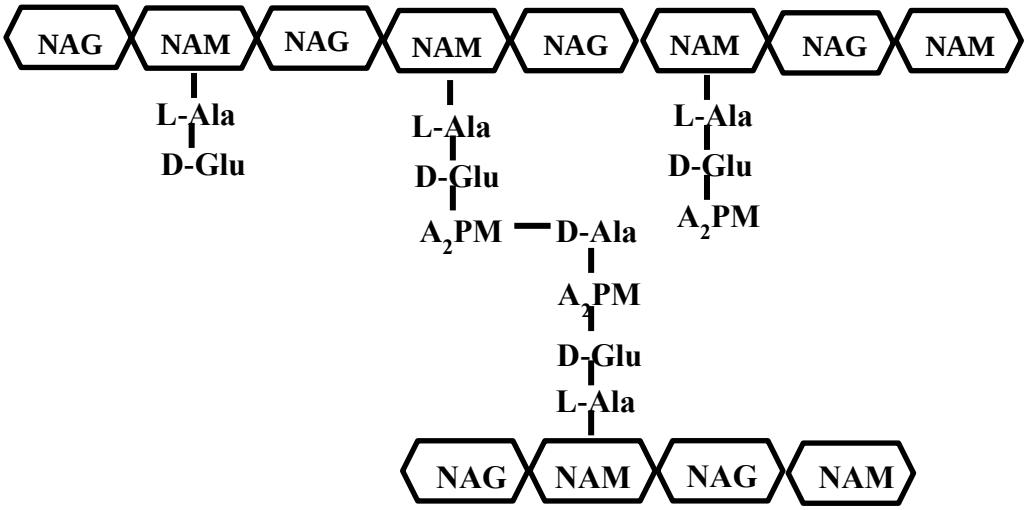


Figure 1.10 Bacterial cell wall structure consist of a backbone having repeated NAG and NAM sugar while NAM sugar has short peptides which is alternating dipeptide, tripeptide and tetrapeptide similarly a parallel backbone tripeptide forms a cross-lining of tripeptide tetrapeptide makes network of cell wall repeatedly attach to parallel backbones.

1.8 Structure of spore cortex peptidoglycan

Spore cortex is responsible for dormancy of spore which maintains the dehydration of spore core which makes it heat resistance. The main event in germination of spores is the depolymerization of the cortex peptidoglycan by lytic enzymes (GSLE). Cortex peptidoglycan of spore consist of alternating N-acetylglucosamine and N-acetyl -muramic acid (NAG and NAM), where NAM residues has branch of either a tetra peptide L-Ala-D-glu-Dpm-D-Ala or L-Ala. Half of the muramic acid do not contain the side chains and modified into muramic lactam (MAL) (Fig. 1.11). This PG chain is cross- linked by tetra peptide side chain forming a peptide bond between the carboxy end of D-Ala and ε-amino end of Dpm (Popham *et al.*, 1996a) the peptidoglycan is cleaved by specific enzymes for specific bonds (Fig. 1.12). Spore peptidoglycan structure is conserved in all bacilli and clostridia. The peptide side chain of N-acetylmuramic acid (NAM) is removed with the help of cwlD-encoded muramoyl-L-Alanine amidase and modified into muramic-δ-lactam (Gilmore *et al.*, 2004).

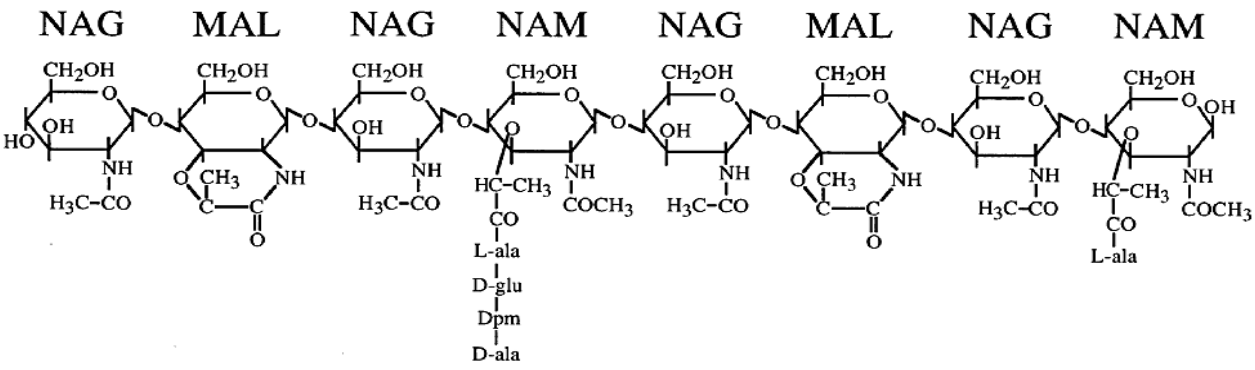


Figure 1.11 Structure of spore cortex peptidoglycan its backbone consists of repeats of NAG, NAM, MAL, with peptides side chains. (Adopted from Popham *et. al.,* 1996a)

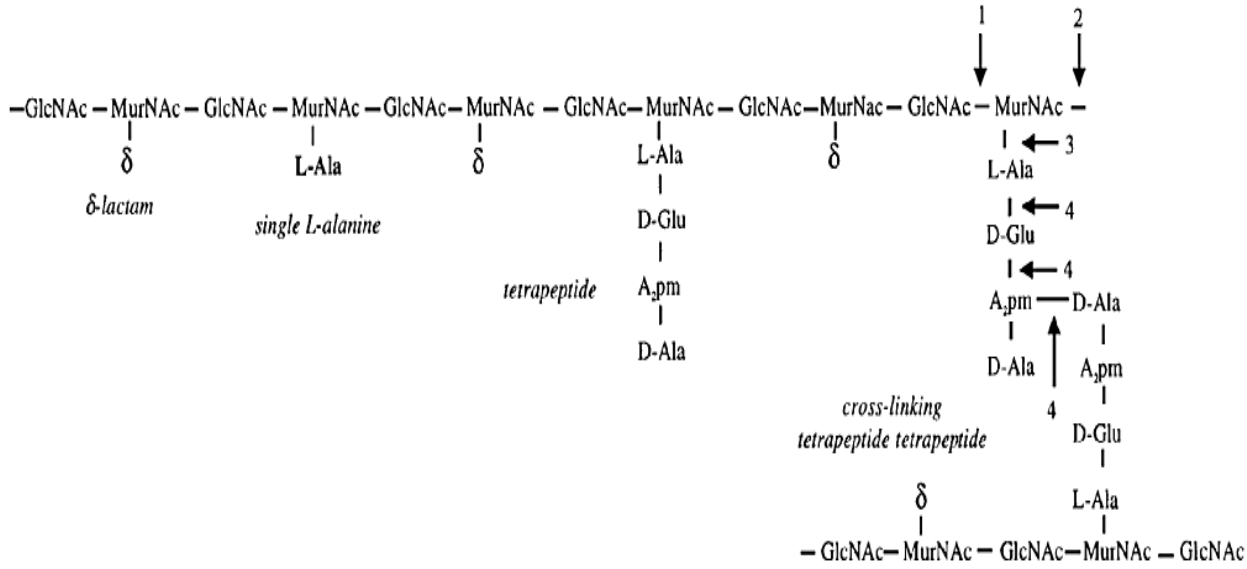


Figure 1.12 Structure of a *B. subtilis* spore cortex peptidoglycan and the cleavage sites with the specific type of enzymes are indicated by arrow (1) Glucosaminidase, (2) muramidases/lytic transglycosylase, (3) amidase, (4) endopeptidase. (Adopted from Smith *et al.,* 2000).

1.9 Autolysin Enzymes (Peptidoglycan hydrolases) involved in bacterial development

In bacteria, there are diverse groups of peptidoglycan hydrolase enzymes which cleaves the bond of the peptidoglycan polymers and its fragments (Shockman *et al.*, 1996). There are hydrolase which cleaves the bond between the cell walls creating pores essential for

incorporation of more PG material and new for expansion of cell wall during cell growth and development of the bacteria (Lee and Huang, 2013).

A cell wall is cleaved with these type of enzymes (Fig. 1.13) (i) Glucosaminidase (ii) Lytic transglycosylase (Lysozymes/ muramidase) (iii) Endopeptidase (iv) Amidases

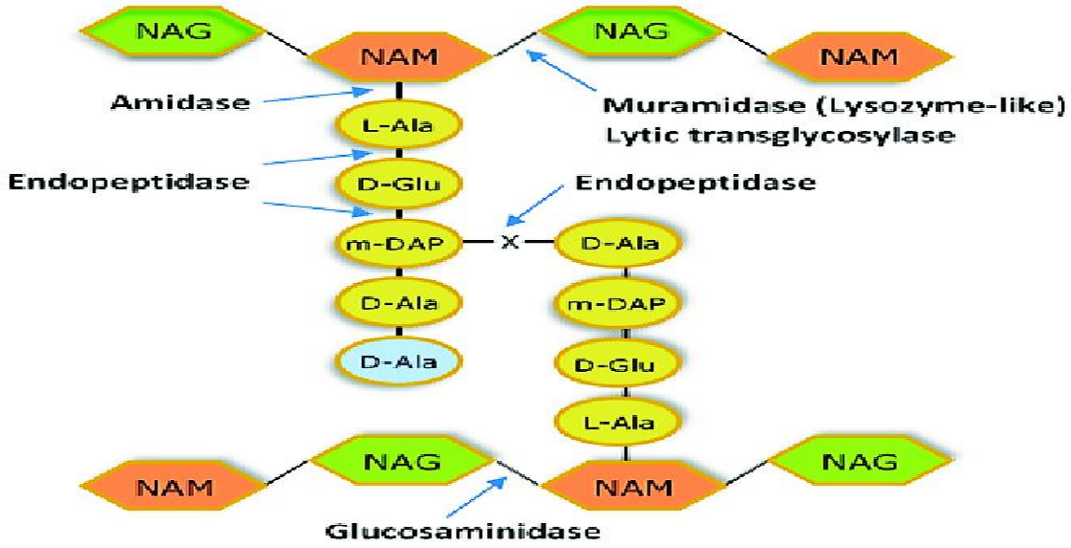


Figure 1.13 Bacterial cell wall peptidoglycan structures with its cleavage sites with different type of enzymes. (Adopted from Fernandes and São-José, 2018)

(i) Glucosaminidase (Lysozymes) - these enzymes cleave the β 1, 4 –glycosidic bond between GlcNAc and MurNAc. They have four classes – Chalaropsis lysozyme, hen egg-white lysozyme (HEWL), goose egg-white lysozyme (GEWL), and bacteriophage T4 lysozyme (T4L).

(ii) Lytic transglycosylase – these enzymes hydrolase the β 1,4 –glycosidic bond between the NAG and NAM. In *E. coli* MltA, MltB(Slt35), MltC, MltD, MltE, MltF and Slt70 function is to recycle peptidoglycan during growth and all have the cleaving activity on the cell wall (peptidoglycan) sacculi like other glycosidase these are not hydrolases (Suvorov *et al.*, 2010).

(iii) Endopeptidase – these cleaves the amide bond between amino acid of alternate peptidoglycan. CwlO and LytE are reported from the *B. subtilis* (Hashimoto *et al.,* 2012). Spr, YebA and YdhO are reported from *E. coli* (Singh *et al.,* 2012) important for cell wall synthesis and its elongation.

(iv) Amidase-N-Acetylmuramyl-L-Ala amidases are amidase which cleaves the amide bond between N-terminal L-alanine residue of peptide and MurNAc. There are four amidases in *E. coli* , AmiA , AmiB, AmiC and AmiD (Heidrich *et al.,* 2001).

The role of these autolysins in vegetative growth, peptidoglycan maturation through the polymerization of peptidoglycan building blocks during these events requires autolysins (Osipovitch *et al.,* 2015). Cell motility, separation and competence, cell-wall turnover, cell enlargement and protein, differentiation into spores and germination, digestion of asymmetric septum for prespore engulfment, maturation of cortex of spore, lysis of mother cell and germination. Germination specific lytic enzymes (GSLE) - These autolysins involve in spore germination like SleB and CwlJ in *B. subtilis* cleaves the spore cortex.

1.10 Germination specific lytic enzymes (GSLE)

GSLE are important enzymes in germination processes they actively recognize the MAL residue in the cortex peptidoglycan of spore and depolymerize the cortex, these enzymes do not cleave leaves the intact cell wall which lacks MAL residue of new emerging bacterial cell just beneath the cortex of spore (Popham *et al.,* 1996b). GSLE are lytic transglycosylase type enzymes these are two types (i) Spore cortex lytic enzymes (SCLEs), hydrolyze the PG cortex of the spore, (ii) Cortical fragment lytic enzymes (CFLEs), these enzymes cleaves the cortex after SCLE hydrolyzes during germination. These both enzymes present in the spore producing members of genera *Bacillus* and *Clostridium* Spp., *B. anthracis* has 3 SCLEs identified SleB,

CwlJ1, CwlJ2, one CFLE is SleL. GSLE allows full hydration and core expansion (Setlow, 2013). *B. subtilis* has three GSLE– CwlJ, SleB and YkvT of these SleB and CwlJ are well studied their double mutants has completely stopped the hydrolysis of cortex (Ishikawa *et al.*, 1998). *Clostridum difficile* and *C. perfringens* spore germination protein is SleC and SleM which are muramidases (Gutelius *et al.*, 2014). In *B. cereus* cortex lytic enzyme is SleL (YaaH) which shows glucosaminidase activity (Chen *et al.*, 2000).

1.11 SleB enzyme

SleB a lytic transglycosylase is well characterized protein, expressed during sporulation as an inactive form and then deposited on the spore outer cortex (Moriyama *et al.*, 1999). During germination the enzyme is activated and hydrolyses the peptidoglycan of cortex. SleB recognize the cortex- specific muramic δ lactam residue which provides the specificity determinant. Sleb crystal structure of its C-terminal domain has transglycosylase fold and its active site having a catalytic glutamate residue (Jing *et al.*, 2012).

1.12 Genome mining

It is the computational process requires bioinformatics tools; one can isolate the gene of interest of a genotype from a related genome. In gene bank database now thousands of bacterial complete genomes are submitted which are annotated and genes are identified and characterized experimentally. When any newly bacterial genome is sequenced, it's all the coding sequences (CDSs) is identified then these CDS can be compared to the CDS present already in the database. Therefore, many known CDS are present in the database such known genes can be used to identify their putative genes in the newly sequenced genome by sequence comparison (Ziemert *et al.*, 2016).

1.13 About *Rhodobacter johrii*

Rba. johrii is an endospore forming phototrophic proteobacterium, isolated from rhizosphere soil (Girija *et al.*, 2010). *Rba. johrii* is a Gram stain negative, oval to rod-shaped, motile, phototrophic, purple non-sulfur bacterium. It is commonly found in flooded paddy soils and has role in nutrient cycle. It has bio fertilizer properties like nitrogenase activity, production of indole - 3 -acetic acid production, nitrogen fixation. Though a few morphological characters and differences in the structure of spore of *Rba. johrii* with respect to that of *Bacillus* and other Gram-stain-positive bacterial spores was studied but still an extensive and more elaborate study is to be done.

1.14 Definition of the problem

Many bacteria other than firmicute groups produce dormant structures when thrives in stressful environmental surroundings (McCaul and Williams, 1981; Ajithkumar *et al*., 2003; Singh *et al.,* 2010; Ghosh *et al.,* 2009; Lamont *et al.,* 2012; Venkidusamy and Megharaj, 2016; Filippidou *et al.,* 2019). Our group has reported similar finding a non-firmicute, gram-stain-negative phototrophic, alphaproteobacterium, *Rba. johrii* which produces endospores like structures under certain conditions. The bacterium was isolated from a pasteurized soil of paddy rice fields in Andhra Pradesh in India (Girija *et al.,* 2010). As is the norm with rice cultivation, these fields undergo periods of flooding and drying and such seemingly “normal” environments may be viewed as “adverse” from the point of view of the micro biota living there (Liesack *et al.,* 2000). Therefore, microorganisms may have a reasonable motive for the formation of dormant structures to cope with survival challenges posed in such habitats. As this bacterium was isolated from the pasteurized soil (heated at 80°C for 30 min), therefore has been revive probably from the dormant bodies. The present work aims in understanding more about

endospores of *Rba. johrii* by examining the spore ultra-structure and molecular insights by identifying the genes involve in spore formation through genome sequencing and gene mining with the following objective :

1.15 Objective of the study

- **To study the insights of endospores and molecular machinery of sporogenesis in *Rba. johrii***

The above objective will be achieved through the following scientific approach:

- ***Endospore like refractive bodies reproducibility & reconfirmation: Phenotypic study***
- ***Genome sequencing and mining of genes involved in sporulation***
- ***Characterization of at least one of the putative protein of spore***

MATERIALS AND METHODS

2. Materials and methods

2.1 Materials, chemical reagents and equipment's

2.1.1 Apparatus

Apparatus required for the present experiments include culturing glass flask, beaker, measuring cylinders, test tubes, reagent bottles, screw cap tubes, screw cap bottles, plastic and glass petri dish, pipettes were used make of Borosil or Riviera or Duran or Anumbra brand.

2.1.2 Deionized water and Milli-Q water

Deionized water was used for media preparation and final rinsing of glassware after washing. Milli-Q water was used for molecular work, these were obtained from the water plant facility unit, school of life sciences, University of Hyderabad, which was further autoclaved and stock solutions were prepared.

2.1.3 Compounds and chemicals

The chemicals which were used in the current research work was from HIMEDIA, Invitrogen, Sigma, Qiagen, Thermofisher scientific, E-Merck.

2.1.4 Adjusting pH

pH meter (Digisun Electronics, DL-707) was used for adjusting the pH.

2.1.5 Sterilization

Growth media, Eppendorf tubes, glass equipments and apparatuses were sterilized through the autoclave for 15 min at 15 lbs pressure. Compounds which are heat sensitive are sterilized through Acrodisc syringe filters with Supor membrane (Hydrophilic polyethersulfone), sterile- pore size of 0.45 µm or 0.2 µm (PALL, Life Sciences).

2.2 Microbiological methods

2.2.1 Growth media

The Biebl and Pfenning, 1981 a mineral medium was modified and used for the growth of *Rba. johrii and R. benzoatilyticus*, all the components are given in table (Table 2.1).

Table 2.1 Composition of modified Biebl and Pfennig (1981) medium

Ingredients	$g.l^{-1}$
KH_2PO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.4
NH_4Cl	0.4
$CaCl_2.2H_20$	0.05
Pyruvate	3.0
Yeast Extract	0.6
Fe-citrate solution (0.1% w/v)	5 ml
*Micronutrient solution (SL7)	1 ml

2.2.2 Growth and sporulation conditions

Rba. johrii was grown at 30°C on modified mineral medium (Biebl and Pfenning, 1981) in the screw capped tubes at 30°C incubation and 2400 lx light intensity. *Rba. johrii* was grown phototrophically (OD_{660} nm= 0.8) till mid log which was inoculated to the Difco sporulation medium (DSM) (Nicholson and Setlow, 1990). After the growth comes till log phase keep at 80°C for 10-15 min and finally keep for 1-2 days at RT. *Rba. johrii* grown in agar plates of mineral medium incubated for 1-2 months and also the old culture in broth grown media were sources of endospore-like dormant structures. Samples are then observed for endospores.

2.2.3 Spore isolation from *Rba. johrii*

For isolation of spore from the *Rba. johrii* a modified protocol was used (Bosak *et al.*, 2008). Briefly, centrifuge the *Rba. johrii* culture at 6,000 rpm for 10 min. Wash the pellet with PBS (pH 7.8) buffer at least 2-3 times, then dissolve in 50 ml Tris EDTA buffer containing lysozyme 1.2 mg/ml and incubate for 1-2 h at 37°C shaker. Add 10 ml SDS and further incubate

at 37°C for 20-30 min then again wash it for 2-3 times. Dissolve the pellet in Milli-Q and store at 4 °C. The resulting yield of spores was found to be 90 % pure.

2.2.4 Phase contrast microscopy

Phase contrast microscope (Olympus-B201) was used to analyse the morphology of cell like cell shape, spore, granules and other intracellular or extracellular structures.

2.2.5 Endospore staining

Staining of induced cultures for spore-like structures was done using malachite green and safranin (Schaeffer and Fulton, 1933). Perform a bacterial smear of *Rba. johrii* culture heat the slide gently over the water bath for 5 min, stain with malachite green over the smear heat the slide gently for 5 min. Remove slide and wash the slide through running tap water, then add the safranin stain for 2-3 min. Again wash with water and dry the slide completely to visualize underneath light microscope (Olympus B201). Endospores will stain green. Parent cells will stain red.

2.2.6 Nile red staining for PHA granules

Staining of cells with Nile red (Sigma) 1% aqueous solution in DMSO (Ostle and Holt, 1982). *Rba. johrii* cells were heat fixed and few drops of Nile red was added on smear kept in water bath at 55°C for 10-15 min. Again wash the slides with running water, then add 8% acetic acid for 2 min followed by washing with water, air dry the slide and place a coverslip. Samples were observed under confocal microscope (ZEISS LSM900) at red excitation wavelengths (filter BP 450-500 nm).

2.2.7 FM 4-64 and Nile red dye counterstaining

The late stationary phase grown *Rba. johrii* culture was centrifuge for 10 min at 10,000 rpm for pelleting. Wash the pellet with Milli-Q and resuspend in 200 µl Milli-Q. Now add 10 µl

FM4-64 from stock of 5 µg/ml and add 5 µl. of Nile red from stock of 1 mg/ml. Incubate the cells in 30°C water bath around 30 min in dark. Again wash the slides with running water and air dry. Place the coverslip and visualize under confocal laser scanning microscopy (ZEISS LSM900).

2.2.8 FM 4-64 and DAPI dye staining

The *Rba. johrii* late stationary phase grown culture was stained with FM 4-64 and DAPI dye (Sigma Aldrich, Inc., USA). 100 µl culture was washed with autoclaved distilled water and pellet was resuspended in 200 µl of autoclaved Milli-Q water. 10 µl of FM 4-64 from a stock of 5 $\mu g.ml^{-1}$ was added to the suspension. 10 µl of DAPI from a stock of 30 mM in phosphate buffer saline (PBS) was added. Kept for incubation at RT for 30 min in dark. Wash the slides with autoclaved Milli-Q water and the cells were placed on a clean glass slide with coverslip for observation under a confocal laser scanning microscope (ZEISS LSM900).

2.2.9 Chemical inhibition of PHA granule synthesis and Nile red staining

Acrylic acid and 4-Pentenoic acid are the known potent inhibitors of PHA granules (Lee *et al.*, 2001). These compounds are added at 5 mM concentration to the *Rba. johrii* cultures grown in modified Biebl and Pfennig's medium at 30°C at all growth phases. After treating culture with the inhibitors at each growth phase, the cultures were stained with the Nile red (Ostle and Holt, 1982) for PHA staining and observed under Leica TCS SP2 Laser scanning confocal microscope at the Excitation wavelength 450-500 nm.

2.2.10 "Sporotan" dye staining of spores

Purified spore from *Rba. johrii* culture and also late stationary phase culture having spores around 1 ml was pellet down by centrifugation (8,000 rpm at 4°C for 5 min). Wash and resuspend the pellet with the autoclaved PBS buffer (pH 7.8) for 3-4 times. Take 100 µl resuspended culture and add 6 µl of 4 mM solution of sporotan dye dissolved in DMSO for 10-

15 min at the room temperature (~30°C). After incubation again centrifuge (8,000 rpm at 4°C for 5 min) and wash 1-2 times in Milli-Q water and then resuspend in ~ 20 µl of Milli-Q water. Now make a smear of suspension on the microscopic glass slide and placed a coverslip over it, also seal it properly. Observe the slide under confocal laser scanning microscope model ZEISS LSM 900 for imaging at excitation wavelength of 405 nm and emission wavelength 410-480 nm.

2.2.11 Nile red staining of chloroform treated *Rba. johrii* culture

About 5 ml of the late stationary phase culture pellet was mixed with 3 ml of chloroform and kept for boiling for 10 min. After boiling the pellet stain with Nile red (Ostle and Holt, 1982). The slide was observed under confocal laser scanning microscope model ZEISS LSM 900 at the excitation wavelength 450-500 nm.

2.2.12 Scanning electron microscopy (SEM)

Rba. johrii cell pellet was fixed with 2.5% glutaraldehyde, then wash in 0.1M PBS 2-3 times. Again fix in 1% Osmium tetroxide and wash in 0.1M PBS for 2-3 times. Now dehydrate the pellet in ethanol (40%, 50%, 70%, 90% and 100% ethanol) and dried on cover slip. Cambridge stubs were used for mounting the cover slip and gold sputtering was done. The sample was observed under scanning electron microscope (JEOL JSM 5600).

2.2.13 Transmission electron microscope (TEM sectioning)

Sample preparation of thin section and TEM observation of *Rba. johrii* was outsourced to the RUSKA Lab, SVVU, Rajendranagar, Hyderabad. Briefly, the samples were fixed with the 2.5% glutaraldehyde in 0.05 M PBS (pH 7.2) at 4°C for 24 h. Again fix in 2% osmium tetroxide for 2 h with same PBS buffer. Samples were embedded into the spurr resin after dehydrating in different alcohol grade. The ultra-microtome was used for ultra-thin sectioning (50-70 nm) and semi thin sectioning (200-300 nm) , these sections were stained with uranyl acetate and toludine

blue. The sections were again stained with 4% lead citrate. Grids were used for mounting both the sections. The grids were observed under TEM (Hitachi, H-7500)

2.3 Bioinformatical (*in silico*) methodology

2.3.1 Genome sequencing and assembly

DNA was extracted using the Quick-DNATM Universal Kit (ZYMO RESEARCH) with utmost care to prevent contamination. Genome sequencing was outsourced to SciGenom Labs Pvt., Ltd., India. Briefly, Covaris system was used for fragmentation of DNA creating the 300-350 bp mean size. Illumina TrueSeq procedure was followed for preparing the DNA shotgun library and Illumina Hiseq platform was used for sequencing. A total 2,959,826 reads were produced from the sequenced DNA library. Optimal K-value predicted was 19 from KmerGenie (Chikhi and Medvedev, 2014), with assembly size 45,19,038 bp. FastQCn (www. bioinformatics. babraham.ac.uk/projects/fastqc) checked the raw reads quality and FastX tool (//hannonlab.cshl. edu/fastx_toolkit) was used for trimming the raw reads. De novo assembly was performed using MaSuRCA (Zimin *et al*., 2013), Velvet (Zerbino and Birney, 2008), Edena (Hernandez *et al.,* 2008), SOAPdenovo2 (Luo *et al.*, 2012), SPAdes (Bankevich *et al*., 2012), ABySS (Simpson *et al*., 2009). Integration of the contigs was done with the CISA (Lin and Liao, 2013).

2.3.2 Annotation and analysis

NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) (Tatusova *et al*., 2016) and RAST server (Aziz *et al.,* 2008) was used for annotations. tRNA and rRNA were predicted using RNAmmer (Lagesen *et al.,* 2007). GGDC web server (//ggdc.dsmz.de) was used for calculating DNA-DNA hybridization *in silico* and ANI calculator (//enveomics. ce.gatech.edu/ani) for calculating average nucleotide identity (ANI). Identification of species

was done with SpecI server. Circular map showing the spore homolog was drawn using CGView Server (Grant and Stothard, 2008).

2.3.3 Genome characterization

To understand more in the insights within the genus *Rhodobacter* Spp. we have employed various online servers as well as offline tools such as RAST (Aziz *et al.,* 2008), PATRIC (Wattam *et al.,* 2017), AntiSMASH (Blin *et al.,* 2019), dbCAN2 (Zhang *et al.,* 2018), CRISPR finder (Grissa *et al.,* 2007), KAAS (Moriya *et al.,* 2007), Pfam (El-Gebali *et al.,* 2019), TMHMM (//cbs.dtu.dk/services/TMHMM/), SignalP (Armenteros *et al.,* 2019), BPGA (Chaudhari *et al.,* 2016) and DNA Plotter (Carver *et al.,* 2009) for circular map visualization.

2.3.4 Genome mining for endospore genes

Non-redundant database of complete proteins of *Rba. johrii* and *Bacillus subtilis* subsp. *subtilis* 168 from NCBI database was downloaded. *Rba. johrii* complete protein orthologs were searched against *Bacillus subtilis* subsp. subtilis 168 genome (Accession No.NC000964) proteins using BLASTp tool using non-redundant protein database of NCBI with Blosum 45 Matrix and other default parameters.

2.3.5 Detection of Conserved Domain and multiple sequence alignment

Using Simple Modular Architecture Research Tool (SMART) (Schultz *et al*., 1998) Full length protein and catalytic domain alignment was done with *B. cereus* strains, ATCC 14579 (NCBI Accession No. WP001249053), Q1 (WP001249040), M13 (ASK14870), MLY1 (WP100910207); *B. anthracis* strains, ATCC 14578 (WP001249045), Sterne (YP028821), Ames (AAP26584), and *B. subtilis* subsp. *spizizenii* strain TU-B-10 (WP014114227), Stercoris (WP014664430), strain 168 (WP004398523) using Clustal Omega (Madeira *et al.,* 2019) and rendered sequence similarity by ESPript 3.0 (Robert and Goue, 2014).

2.3.6 Protein 3D secondary structure model and its validation

The SWISS MODEL (Waterhouse *et al.,* 2018) and RaptorX server (Kallberg *et al.,* 2012) was used to generate structural model of the SleR. The model obtained from SWISS-MODEL was verified using PROCHECK (Laskowski *et al.,* 1993). Structural diagrams visualization is done by PyMOL. (/pymol.org/educational).

2.3.7 Superimposition and molecular docking

Protein model SleR were superimposed based on Cα position by PyMol Molecular graphics system with crystal structure of *B. cereus* ATCC 14579 SleB protein and crystal structure of SleB from *B. anthracis*. To find substrate interaction with SleR from *Rba. johrii,* molecular docking was performed with 3D coordinates of N-Acetylglucosamine, N-Acetylmuramic acid, NAG-NAM-(Pentapeptide), Muramic Lactam, 1,6-anhydromuropeptide (PDB no. 1QTE) (Van Asselt *et al.,* 1999), NAM-NAG-NAM (NAM and NAG alternating trisaccharide) (PDB no. 9LYZ) (Kelly *et al*., 1979). These substrates were taken from crystal structures from protein data bank. AutoDock Vina (Trott and Olson, 2010) was used for the docking and PyRx for visualization (Dallakyan and Olson, 2015). Selected substrates were docked manually into the catalytic domain of the SleR of *Rba. johrii*. Grid box was generated around the protein with grid box size of center X=55.09, Y=45.61, Z=27.22 and dimension at X=33.31, Y=45.3, Z=38.01 and exhaustiveness 8 was calculated. Molecules having high docking score and preferable binding interactions were analyzed using Discovery Studio 2017 R2 Client and PyMOL graphics viewer.

2.4 Protein characterization

2.4.1 Bacterial strain, growth conditions and plasmids

Rhodobacter johrii strain JA192 (= DMS 18678 = JCM 14543 = MTCC 8172) was grown in a modified mineral medium (Lakshmi *et al.,* 2011). *B. subtilis* FW2 was inoculated in

the nutrient media (Himedia M002) and incubated overnight at 37°C shaker for sporulation. *E. coli* DH5α and Rosetta (DE3) were grown using lurea broth media (Himedia M1245) at 37°C and the antibiotic used was kanamycin at 34 µg/ml concentration. The plasmid used for this study was pET SUMO vector. All cultures were maintained in aseptic conditions with sterility precautions to ensure absence of contamination.

2.4.2 Genomic DNA isolation

Rba. johrii genomic DNA was extracted using the Quick - DNA^{TM} Universal Kit (ZYMO RESEARCH) as guided by manual directions.

2.4.3 Polymerase chain reaction (PCR) amplification of *SleR* gene

For *sleR* gene amplification DNA was extracted from the *Rba. johrii* culture by kit method. Primer (Table 2.2) was design to amplify the *sleR* gene ORF lacking starting 23 codons, including codons 24-240 from *Rba. johrii* genomic DNA. The reaction mixture was set up by using Taq DNA Polymerase (Gene i PCR Master Mix) for PCR; it was prepared as follows (Table 2.3). The programme was set for the PCR amplification (Table 2.4). The amplified product was observed on 1% TBE agarose gel and visualize in Gel Doc (Alpha imager 1220).

Table 2.2: Primer characteristics

Primer	Sequence (5'-3')	T_m	GC%	Annealing temperature
Forward primer (BSCLFSUMOE)	ATATGGATCCGATGTGACGGTGAG C CAGTC	57°C	55.2	52.0^{0}C
Reverse primer (HSCLRSUMOE)	TATAAAGCTTTCAGGAGCCGCCGG GCTG	55.1°C	55.5	50.0^{0}C

Table 2.3: Components of PCR reaction mixture

S.No.	Component	Stock Concentration	Actual Volume used
1.	Nuclease free water		5.5 µl
2.	Master Mix	2X	12.5 µl
3.	Forward Primer	10 µM	1 µl
4.	Reverse Primer	10 µM	1 µl
5.	Template DNA (colony in MQ)		5 µl
	Total Volume		25 µl

Table 2.4: Programme for PCR

1 cycle	94°C for 5 min	Initial denaturation
30 cycles	94°C for 1min	Denaturation
	51°C for 1.5 min	Annealing
	72°C for 1 min	Extension
	72°C for 10 min	Final extension
	Hold at 4°C	

2.4.4 DNA agarose gel electrophoresis

Evaluation of quality of DNA was done by agarose gel electrophoresis using tris-borate EDTA buffer (TBE) at 10 Volt/cm in TBE buffer with ethidium bromide 0.01% (v/v). 6x sample buffer (50 mM EDTA, 30 % (v/v) glycerol, 10 Mm Tris-HCl pH 7.5, 0.28 µM xylencyanol, 0.44 µM bromophenol blue, 8.8 µM orange G) was added to samples to around 1.5 µl to 5 µl of DNA sample and mixed loaded onto the equilibrated TBE gel. Gel was electrophoresed at 50-100 V, visualized and photographed using alpha imager 1220 gel documentation system. DNA fragments were cut out from the gel and isolated with the help of kit (QIAquick Gel Extraction KiT

2.4.5 Molecular gene cloning of truncated *sleR*

PCR amplified product 674 bp of truncated *sleR* and pET SUMO expression vector was

digested with BamHI and HindIII restriction enzyme and purified (QIAquick PCR purification Kit; Qiagen). Ligation is done of PCR product and pET SUMO expression vector by using T4 DNA ligase (Invitrogen) (Table 2.5) followed by transformation into *E. coli* DH5α cells and successful cloned colony plasmid was confirmed by DNA sequencing of the $SleR_{24-240}$ fused with pET SUMO producing the plasmid pET-SUMO-$SleR_{24-240}$, which will encode an N-terminal His_6 - SUMO tagged with SleR truncated fusion protein.

Table 2.5 Ligation of digested *sleR* and pET SUMO vector

Components	**Conc.**	**Final vol.**
Ligase reaction buffer	5x	6 µl
PCR product	150 ng.	2.7 µl
pET SUMO vector	50 ng.	3 µl
T4DNA ligase	0.1 unit	1 µl
Nuclease free water		17.3µl
	Total	30 µl

Total final volume was 30 µl ligation reaction mixture was incubated at 22°C overnight.

2.4.6 Transformation into *E. coli* (DH5-α strain) for cloning

Partially thawed competent cells (DH5-α strain) 100 µl were kept on ice and ligation mix (20 µl.) was added. Incubate for 10-15 min on ice. Heat shock was given at 42°C around 55 s again place into ice around 2 min. Add around 850 µl of lurea broth, then incubate the eppendorf in 37˚C incubation for 1 h at 150 rpm, then centrifuge at 6000 rpm for 10 min and discarded around 800 µl of supernatant, mix the pellet with left over supernatant properly through pipette, plates of luria agar with antibiotic kanamycin were prepared, spread the mix on plates and incubate at 37°C for overnight. Colonies appeared on plates containing recombinant clones after overnight incubation.

2.4.7 Analysis of recombinant clones by PCR of colony and plasmid isolation

From the overnight transformed plate some white colonies were picked and DNA was extracted (Englen and Kelley, 2000) and plasmid isolation was done by QIAGEN kit. The reaction mixture and reaction conditions are same as above described for *SleR* gene amplification.

2.4.8 Plasmid isolation of recombinant colonies

Colonies as appeared on agar plates containing Ampicillin was inoculated in to 10 ml luria broth culture, for its plasmid isolation by QIAprep kit. Plasmid isolated is analysed on 1% agarose gel on electrophoresis and run at 50 v after completion it is analysed by UV doc.

2.4.9 Double digestion

Plasmid isolated from positive colony was checked for the insert *sleR* gene by double digestion. Bam HI and Hind III restriction enzyme were used for double digestion. Take 1 µg of plasmid, add 10 x buffer, 1 µl each of Bam HI and Hind III enzyme make up the total volume 50 µl reaction with Milli-Q. Incubate at 37°C for 3 h. Check and visualize the reaction on 1.2 % agarose gel.

2.4.10 Transformation into *E. coli* Rosetta (DE3 strain) for expression

Partially thawed the competent cells *E. coli* Rosetta (DE3) 100 µl. on ice and add 20 µl. pET- SUMO- $SleR_{24\text{-}240}$ plasmid, incubate for 10-15 min on ice. Heat shock was given at 42°C around 55 s again place into ice around 2 min. Add around 850 µl of lurea broth, then incubate the eppendorf in 37˚C incubation for 1 h at 150 rpm for 1 h centrifuge at 6000 rpm for 10 min and discarded around 800 µl of supernatant, mix pellet with left over supernatant properly through pipette, plates of luria agar with antibiotic kanamycin were prepared, spread the mix on

plates and incubate at 37°C for overnight. Colonies appeared on plates containing recombinant clones after overnight incubation.

2.4.11 Protein overexpression

His_6-SUMO-$SLER_{24-240}$ fusion protein was overexpressed into *E. coli* Rosetta (λDE3) which was grown into 1 L LB medium with 34 µg/ml kanamycin at 37 °C till the OD reaches 0.6, at this point add 0.2 mM of IPTG was added in the culture and kept for incubation for 6 h at 37°C.

2.4.12 Protein purification

Cells were harvested by centrifuging for 10 min at 6,500 rpm, 4°C and dissolved in buffer A (Tris base (pH 8.0) 50 mM, NaCl 300 mM , imidazole 10 mM, PMSF 0.5 Mm) and cell disruption was done by sonication (35% amplitude, 10 min processing time, 20 s intervals) after that cell lysate was pelleted by centrifuging (7,000 rpm for 30 min at 4°C) and supernatant containing the fusion soluble homogenized protein with his tag was purified by metal affinity chromatography with the Ni-NTA resins, elution of proteins was done in buffer A containing 100 to 400 Mm imidazole gradient, fractions with eluted proteins were pooled together, electrophoresed through SDS-PAGE gels and the appropriate band was excised and sent for MALDI-TOF MS-MS sequencing, outsourced to Sandor Life Sciences, Hyderabad, India. The pooled fractions were dialyzed in buffer with SUMO protease without imidazole and passed again through Ni-NTA resins which have yield pure $SLER_{24-240}$ protein and Amicon filter device (Millipore) was used for concentrating the protein.

2.4.13 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Purity and expression of protein was checked on the SDS-PAGE (Schagger and von Jagow, 1987; Laemmli, 1970) at denaturing conditions. First the SDS gel was prepared of stalking and resolving gel according to the required components (Table 2.6) Take the samples of

purified protein or crude cell lysate add equal amount of 4X Laemmli buffer (Tris-Hcl (pH 6.8) 0.15 M, glycerol 20% , SDS 4%, bromophenolblue 0.02 %, β-mercaptoethanol 10%) then heat at 98°C for 6 min before loading on gel. Protein molecular weight was estimated with the electrophoresis in Bio-Rad chamber containing 1X running buffer (Tris 25mM, 0.1% (W/V) SDS, Glycine 190 mM) at 100-150 V. When the dye reaches at near the bottom of resolving gel electrophoresis is switched off and the gel is soaked in stain (10% acetic acid, 25% methanol, 0.25% coomassie brilliant blue R250) for 1 h and then kept in destain (10% acetic acid, 25% methanol) till the protein appear visually clear.

Table 2.6 Components for preparing sodium dodecyl sulphate polyacrylamide gel.

Components	**Resolving gel (10 ml.)**		**Stacking gel (5 ml.)**	
	Volume	Final conc.	Volume	Final conc.
Water	3 ml	-	2.9 ml	
1.5 M Tris Hcl (pH 8.8)	2.5 ml.	375Mm	-	-
10%(W/V) SDS	0.1ml.	0.1%	0.1ml.	0.1%
0.5 M Tris Hcl (pH 6.8)	-	-	1.25 ml.	125 mM
30% acrylamide / bisacrylamide	4.3 ml.	12.9%	0.825 ml.	4.95%
TEMED	6 µl.	0.06%	3.75 µl.	0.75%
10%(W/V) APS	80 µl.	0.08%	50 µl.	0.1%

2.4.14 Western blotting

Total protein of *E. coli* cell lysate was separated on 6-12% SDS-PAGE and transported to the nitrocellulose membrane. Incubate the blot to block with 5% BSA for 1-2 h at RT, then incubated with primary antibodies at 4°C. Anti-6-His antibody (Sigma-Aldrich Inc., USA) was used as primary antibody. Specific protein interacts primary antibodies which are detected through peroxidase coupled secondary antibodies. Wash the blots 2-3 times using TBS-T buffer after primary antibody treatment incubate for 1 h in secondary antibody. Again wash with the

same buffer and chemiluminescent solution ECL substrate was used for developing protein blot and Chemidoc system for visualization.

2.4.15 Cell wall (peptidoglycan) and isolation of spore sacculi

Vegetative cell wall (peptidoglycan) from *B. subtilis* (Sigma Aldrich, Inc., USA) was procure and used as substrate for enzyme assay. For spore cortex (peptidoglycan sacculi) it was isolated as follows, first the spores were isolated from *B. subtilis* FW2 as previously described (Bosak *et al.,* 2008) from which spore sacculi was isolated (Dowd *et al.,* 2008) with some changes. Briefly, the harvested spore pellet from 300 ml of *B. subtilis* FW2 was treated with 35 ml solution containing the 1% SDS, 50mM Tris Hcl (pH 7.5), 50 mM DTT. This was boiled for 20 min and washed with warm Milli-Q water till SDS was removed and centrifugation was done for 10 min at 10,000 rpm. Pellet was dissolved in 1ml of mixture having 20 mM $MgSO_4$, 100 mM tris Hcl (pH 7.5), 50 µg RNAse A, 10 µg DNase I and incubated for 2 h at 37°C. To this 100 µg trypsin (TRT PCK, Worthington) in 10 mM CaCl2 was added and incubated at 37°C overnight. The solution was boiled with 1% SDS and washed in Milli-Q water till SDS was removed and dissolved in Milli-Q water for analysis (stored at 4°C).

2.4.16 Enzyme activity assay

Spore sacculi were dissolved in 1 ml solution having 1 mM EDTA, 30 mM $NaPO_4$(pH 7) and 1mM DTT in a cuvette. Enzyme assay was done by observing the % OD_{600} loss/min (Path length of 1 cm) initial OD_{600} was kept 0.2 of sacculi in which protein $SleR_{24\text{-}240}$ from *Rba. johrii* was added at 20 nM concentrations and incubated at 25°C. The absorbance at 600 nm was recorded at each 5 min time interval after agitation for 40 min. Similarly for the substrate cell wall (peptidoglycan) enzyme assay was done with protein $SleR_{24\text{-}240.}$ Enzyme assay was done in triplicates.

2.5 Real - time quantitative polymerase chain reaction (qRT-PCR) analysis

2.5.1 Growth of *Rba. johrii* and determination of growth phases

Rba. johrii was grown at 30°C on modified mineral medium, in the screw capped tubes in 30°C incubation and 2400lx light intensity. Growth phases were determined by growth curve. The growth of *Rba. johrii* was monitor with change in OD and colorimeter (Systronics model 112) at 660 nm (filter 8) was used for measuring the OD against the blank medium.

2.5.2 RNA isolation and cDNA synthesis

The *Rba. johrii* culture was pellet down by centrifuging (8,000 rpm for 5 min at 4°C) in each growth phases. 10 ml culture pellet was used for total RNA isolation through miRNeasy kit (Qiagen) as instructed. DNase I (Qiagen) treatment was given for the removal of DNA. cDNA was synthesis from 1 µg of RNA through Easy Script cDNA kit (Abm) in 10 µl reaction.

2.5.3 Real-time quantitative polymerase chain reaction (qRT-PCR) analysis

The obtained first strand cDNA was amplified using SYBR green FAST Qpcr Master Mix (Kappa Bio systems) in a 10 µl reaction mixture with target RNA specific primers on StepOne Realtime PCR detection system (Applied Bio systems). Primer design and specificity was done through Geneious Primer 3 for qRT-PCR (Table 2.7). qRT-PCR programme conditions were optimize and used (Table 2.8). Melting curve examined the products specificity and each sample was tested in triplicate. The expression levels of the putative *sleR* gene was intended through standard curve method, Ct values for *sleR* gene was normalized to the Ct values obtained from the amplification of housekeeping gene 16S rRNA gene and relative quantification was done by comparative ΔΔCt method (2^(-ΔΔCt)).

Table 2.7 Primers used for qRT- PCR

Primer	Sequence
Forward primer SleR	5'GTA GAA GAG ATG CGC GCC GAT GG 3'
Reverse primer SleR	5'CGGGCAGTTCTCCTTCATGTTCGAC 3'
Forward primer 16S rRNA	5'GATCCTGGCTCAGAATGAACGCTGG 3'
Reverse primer 16S rRNA	5'TCAAACCAGCTATGGATCGTCGGC 3'

Table 2.8 qRT-PCR programme conditions

1 cycle	95°C for 10 min	Initial denaturation
42 cycles	95°C for 15s	Denaturation
	62°C for 30s	Annealing
	72°C for 30s	Extension
	72°C for 10 min	Final extension
	Hold at 4°C	

2.5.4 Staining and microscopy

Phase contrast microscope (Olympus-B201) was used to analyse the morphology of cell like cell shape, spore, granules and other extracellular structures at different growth phases parallely during the *sleR* gene expression study. Staining of cultures for spore structures was done using malachite green and safranin (Schaeffer and Fulton, 1933).

RESULTS

3. Results

3.1 Endospore properties of *Rba. johrii* and other characterizations

3.1.1 Morphological study

3.1.1.1 Confirmation of endospores of *Rba. johrii*

Cells of *Rba. johrii* are oval to rod shaped, having 1-1.5 µm width and 2.5-3µm length (Fig. 3.1A,B). Late stationary phase cells of photoheterotrophically grown cultures of *Rba. johrii* produce endospore like refractile bodies (Fig. 3.2A,B) which stained with malachite green (Fig. 3.3).

3.1.1.1.2 Endospore staining with DAPI and FM 4-64 flouresence dyes

The late stationary phase grown cells were stained with the dual dyes DAPI and FM4-64. All the vegetative cells were stained with DAPI and FM 4-64 dye, while the refractile bodies were stained only with the DAPI dye (Fig. 3.4) confirming that endospore like refractile bodies having the DNA and it is not an artifact.

3.1.1.2 Confirmation of the endospore like refractile bodies which are not mistaken to poly-β-hydroxyalkanoate (PHA) granules

Poly-β-hydroxyalkanoate (PHA) granules accumulate as a food reserve, which appears similar to the endospores under the microscope therefore it was necessary to confirm that the endospore like refractile bodies are not mistaken with the PHA granules.

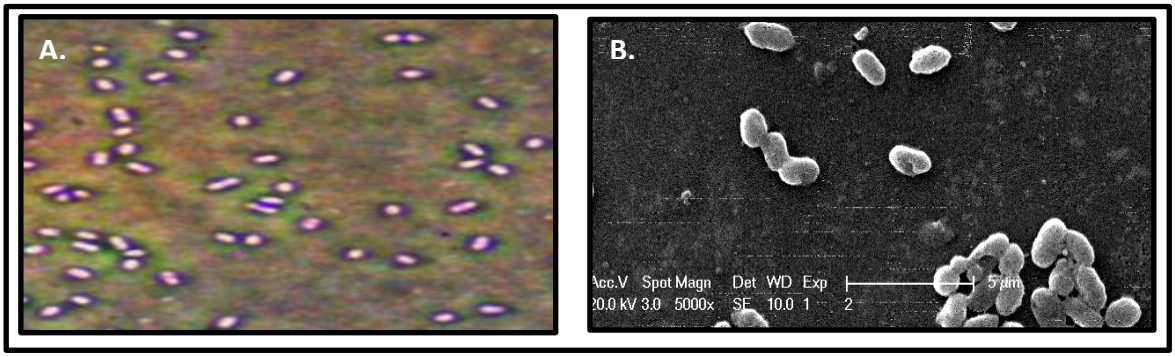


Figure 3.1 Cells of strain *Rba. johrii* A. Phase contrast image B. Scanning electron microscopy image.

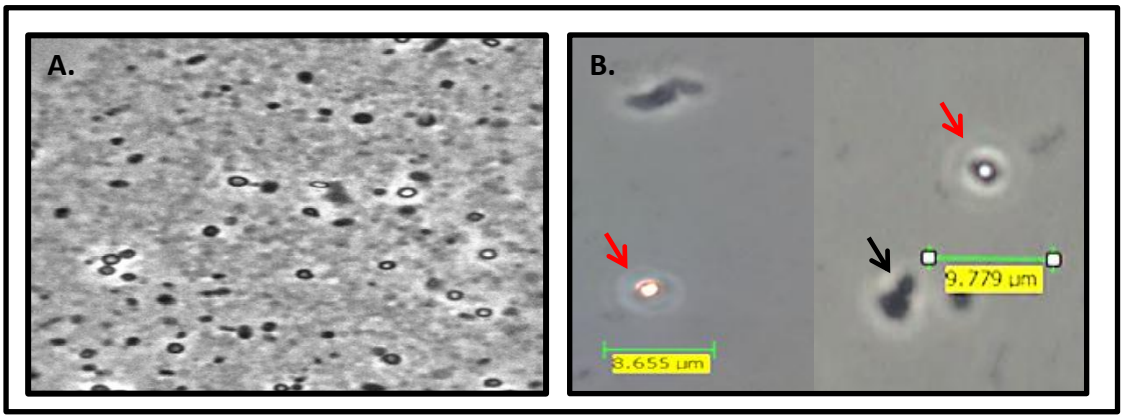


Figure 3.2 Phase contrast image of spores A. Spores in *Rba. johrii* culture B. refractile bodies indicates spore with red arrow and black arrow represent vegetative cell.

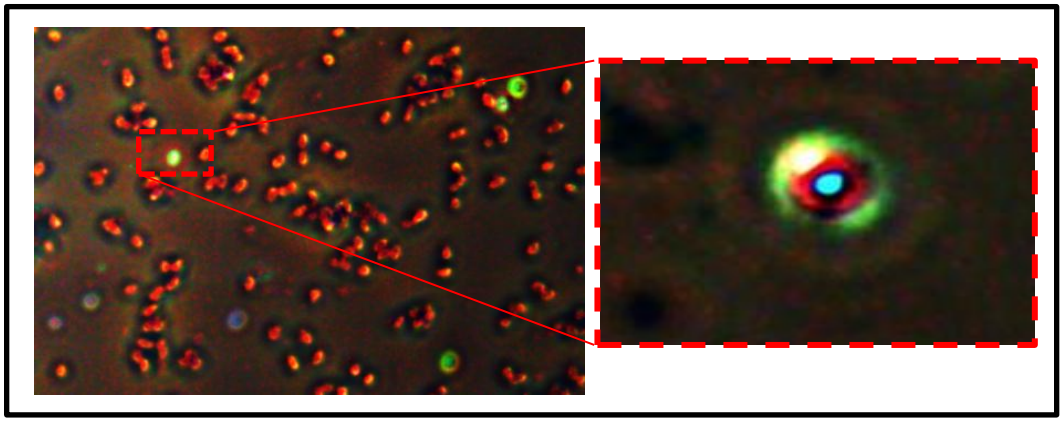


Figure 3.3 Culture stained with malachite green and saffranin.

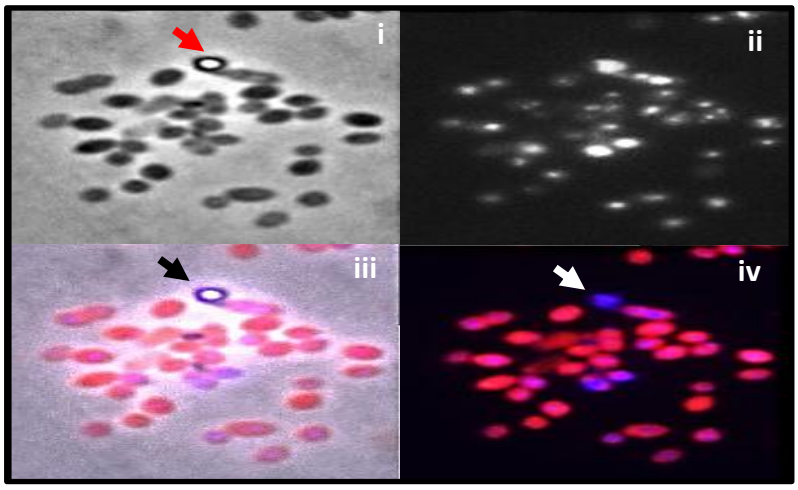


Figure 3.4 Confocal images of sporulated culture (i) Phase contrast micrograph (ii) FM 4-64 stained fluorescent image (iii) overlay of DAPI & FM 4-64 (iv) overlay of phase contrast micrograph, DAPI and FM 4-64.

3.1.1.2.1 FM 4-64 and Nile Red staining

To differentiate between the endospores and PHA granules of *Rba. johrii*, cells were stained with the dual dye FM 4-64 and Nile red. The highly refractile spore like bodies were not stained with the Nile red and PHA granules has red fluorescence (Fig. 3.5). Therefore, we conclude that the late stationary phase cells of *Rba. johrii* produces both the endospores and the PHA granules parallelly and using dual dyes these two bodies are clearly differentiated which confirms that the endospore is not mistaken with PHA granules.

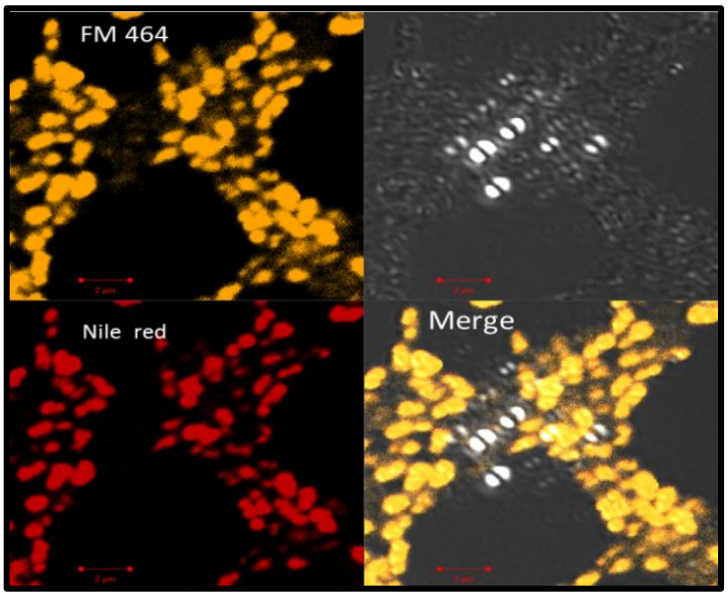


Figure 3.5 Confocal images of sporulated culture stained with Nile red and FM 4-64 flouresence dye.

3.1.1.2.2 Chloroform treatment to dissolve PHA granules

The phototrophically grown cells of *Rba. johrii* were treated with chloroform to dissolve PHA granules. The treated cells were stained with malachite green which showed the green stained spore bodies (Fig. 3.6A). To crosscheck whether still PHA granules are present in the cells after treatment, Nile red staining was done and no fluorescence was observed (Fig. 3.6B) indicating absence of PHA granules.

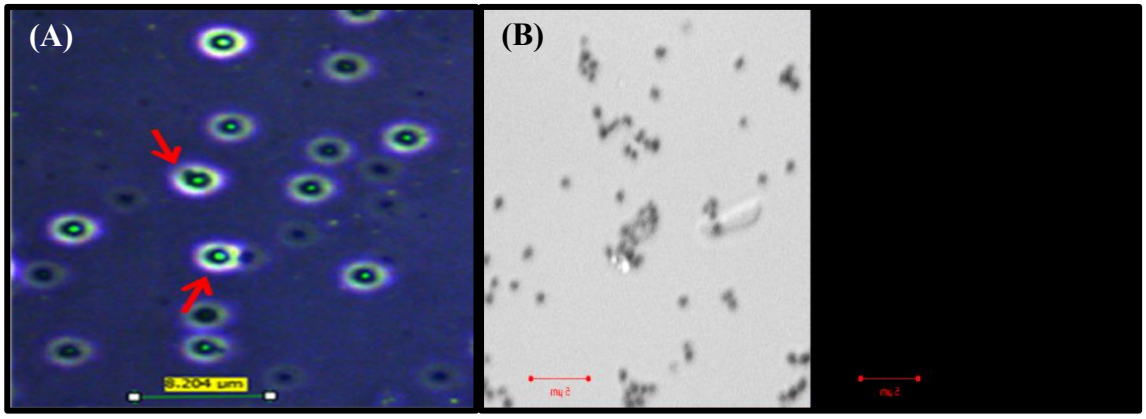


Figure 3.6 (A) Chloroform treatment confirm the refractile bodies as spores and not PHA granules after staining with malachite green (B) Nile Red staining showing no flouresence thus absence of PHA granules.

3.1.1.2.3 Inhibition of PHA biosynthetic pathway

To inhibit the PHA biosynthetic pathway the inhibitors acrylic acid and pentenoic acid were added at different growth phases. At mid log phase, 5 mM of acrylic acid or pentenoic acid was added separately and incubated the culture till late stationary phase. Cells stained with Nile red showed no flouresence (Fig. 3.7A) confirming the inhibition of PHA granules. The untreated cells of *Rba. johrii* culture taken as a positive control which has red fluorescence (Fig. 3.7B) shows presence of PHA granules.

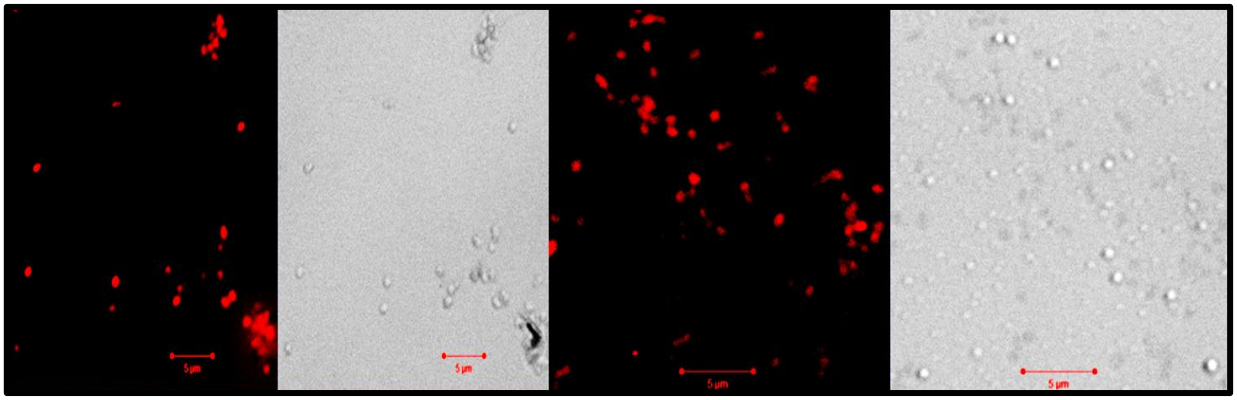


Figure 3.7 (A) PHA granules stained with Nile red and no inhibitors in *Rba. johrii* culture initiated for Spores.

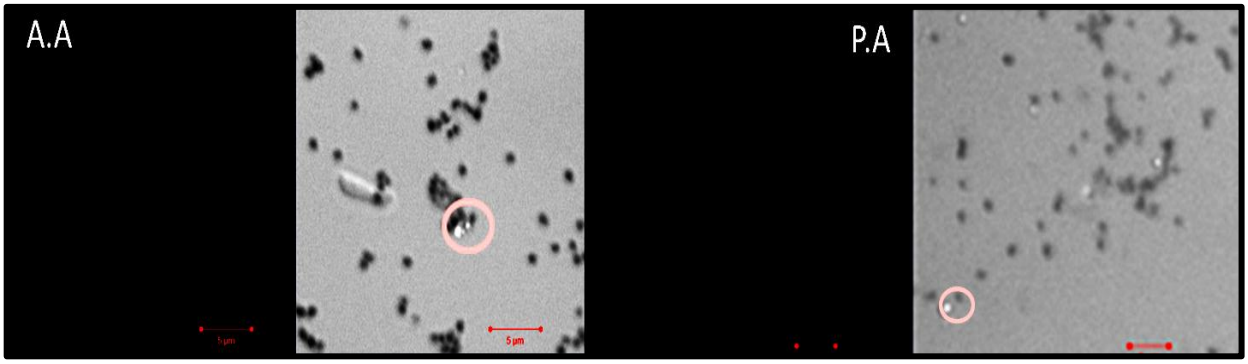


Figure 3.7 (B) PHA granules stained with Nile red and having inhibitors acrylic acid and pentenoic acid in *Rba. johrii* culture initiated for sporulation.

3.1.1.3 Ultra structure of *Rba. johrii* culture and endospores

Ultra-thin sections of *Rba. johrii* cells showed the vegetative cells (V), intracytoplasmic membrane (ICM) structures along with PHA granules (PHA) (Fig. 3.8.1). The ultrafine structure of the dormant spore sections had central core (CR) which was surrounded by two layers inner one is inner thick membrane (IM) and outer membrane like outer coat (OM) in between these two layers is cortex (CX) here inner coat cannot be seen in these dormant bodies and prosthecae like structure were present (Fig. 3.8.2). When the cells of *Rba. johrii* were treated with the Triton –X, the spores coat and other proteinaceous structures were deformed due to denaturation of the protein (Fig. 3.8.2C).

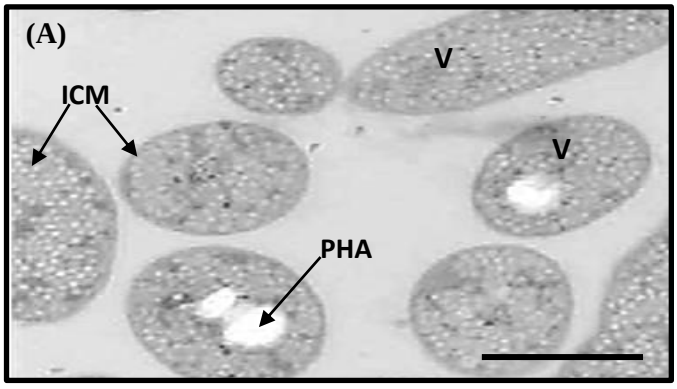


Figure 3.8.1 TEM sectioning images of *Rba. johrii*, (A)Sectioning of vegetative cells (V), vegetative cell with Poly-β-hydroxyalkanoate granules (PHA), endospore like dormant structure (ES), and small intracytoplasmic photosynthetic membrane within vegetative cell (ICM).

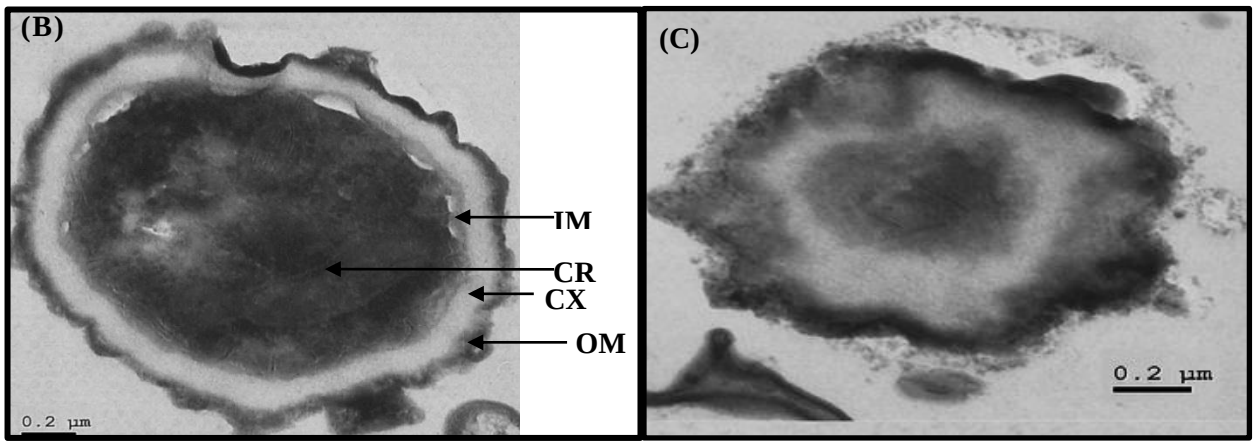


Figure 3.8.2 TEM sectioning images of *Rba. johrii* (B)Ultrafine sections of dormant structure having core (CR), cortex (CX), inner thick membrane (IM), outer membrane like outer coat (OM) and prosthecae structure (PS) (C) section of spores when treated with Triton-X.

3.1.2 A new bacterial spore specific fluorescent stain was used for *Rba. johrii* spore identification.

3.1.2.1 Increasing the number of spores with new method

The photoheterotrophically grown cultures of *Rba. johrii* in Biebl and Pfennig's medium normally develops around 35% of spores (Fig. 3.9A). Therefore, to increase the number of spores many different physical conditions along with the media were screened and finally a method and media was developed, which increased ~20 % yield in the sporulation (Fig. 3.9B).

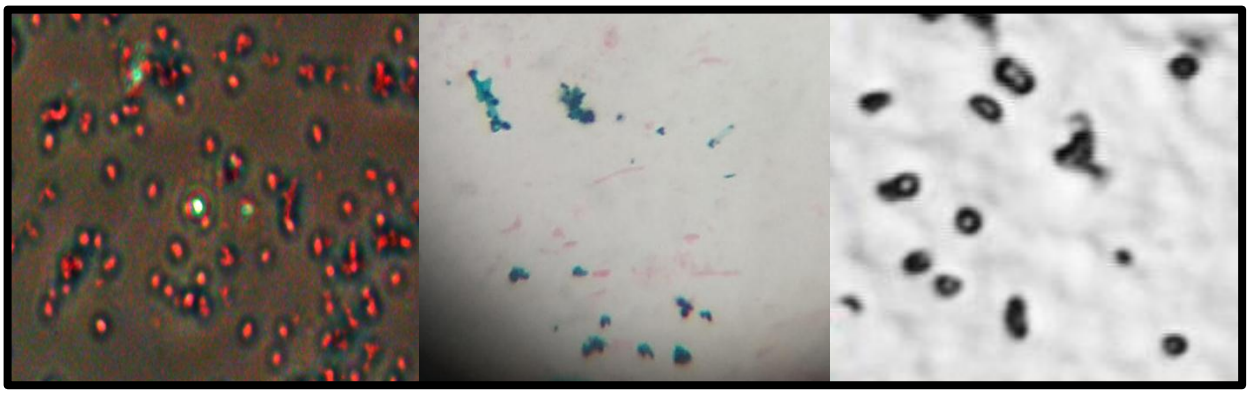


Figure 3.9 (A) Natural endospores in old culture stained with malachite green (B) Artificially induced spores stained with malachite green (C) Isolated pure spores of *Rba. johrii.*

3.1.2.2 Isolation of spores

A modified method was developed to isolate the spores from the *Rba. johrii* given in material and method, which yield the 90 % pure spores free of cell debris and other contaminants (Fig. 3.9C).

3.1.2.3 "Sporotan" staining the spores of firmicute and non-firmicute members

A chemical 7,7-(n-hexylamino)-8,8-dicyanoquinodimethane (Fig. 3.10) which was synthesize chemically was checked for staining the spores of *Rba. johrii.* The staining method developed was very simple, which stains the spores of *Rba. johrii* with the fluorescent dye. "Sporotan" was very specifically binding to the spores but not to the vegetative cells (Fig. 3.11A). Sporotan stains the isolated pure spores of the *Rba. johrii* (Fig. 3.11B). As well as the spores of *B. subtilis* was taken as a positive control (Fig. 3.11C).

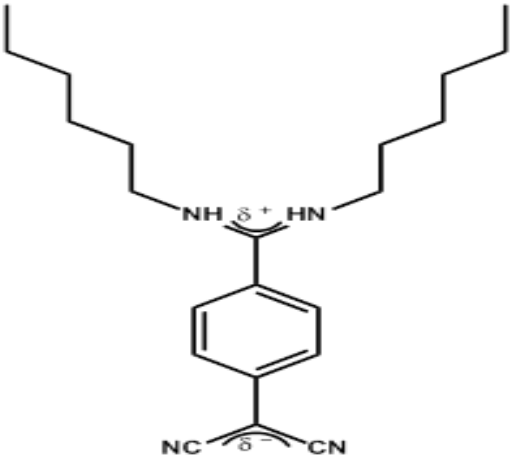
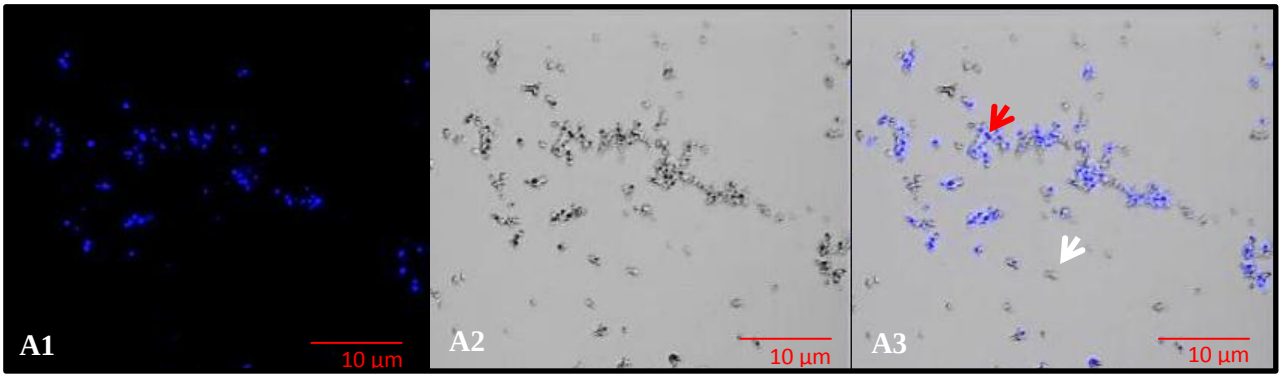
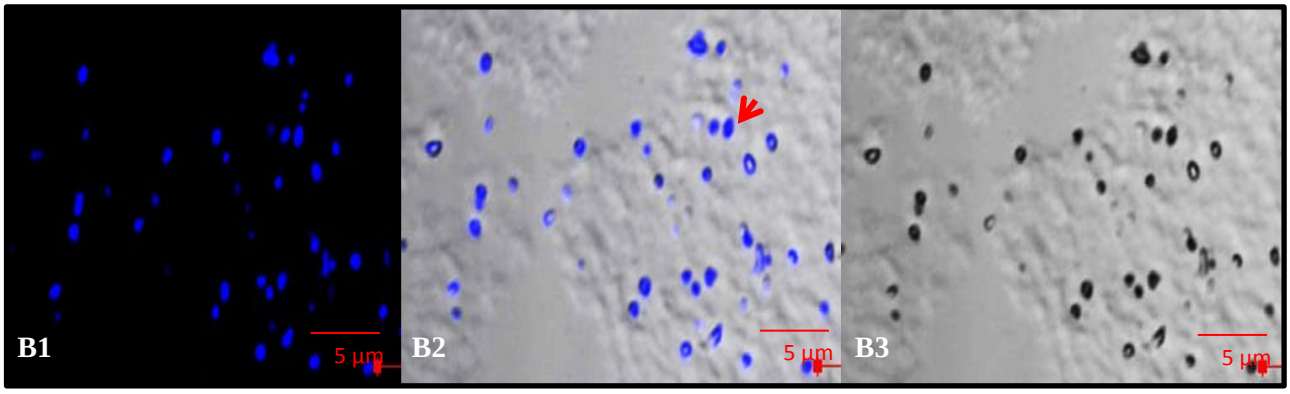


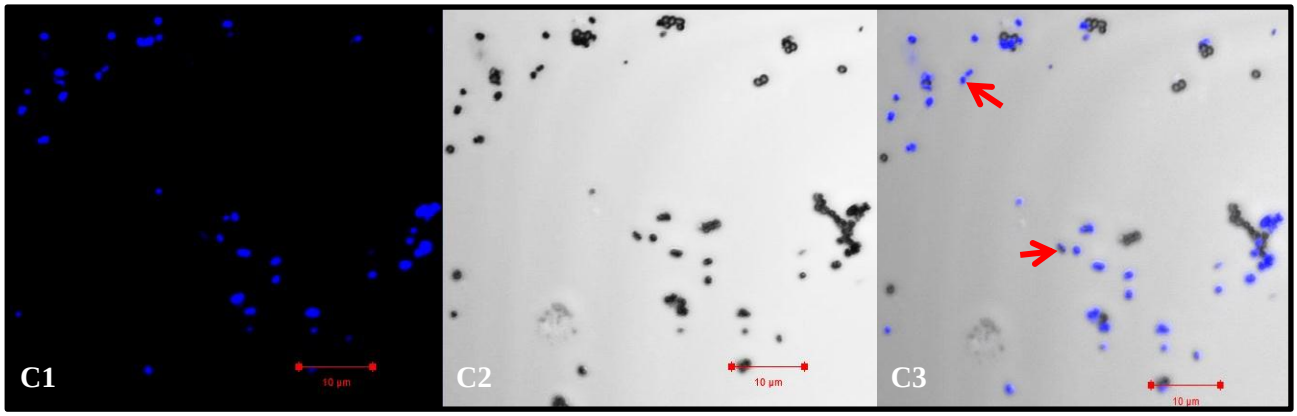
Figure 3.10 Structure of sporotan dye " 7, 7-Bis (*n*-hexylamino)-8, 8-dicyanoquinodimethane".



(A)



(B)



(C)

Figure 3.11 Sporotan staining (A) vegetative cells and spores of *Rba. johrii* stained with "Sporotan" (A1, Fluorescence image; A2, Bright field image; A3, Overlay image) (B) Isolated spores of *Rba. johrii* stained with "Sporotan" (B1, Fluorescence image; B2,;Overlay image B3, Bright field image) (C) Isolated spores of *B. subtilis* stained with "Sporotan"(C1, Fluorescence image; C2, Bright field image; C3, Overlay image) spores are shown in red arrow; White arrows show vegetative cells.

3.1.3 Microcolonies as indicators of spores of *Rba. johrii*

Three months old plates with colonies of *Rba. johrii* when observed under invert microscope. The parent colonies were almost dry and showed microcolonies (Fig. 3.12) which were very similar having colony morphology of *Rba. johrii*. Cells from the microcolonies were also stained with the malachite green (Fig. 3.12).

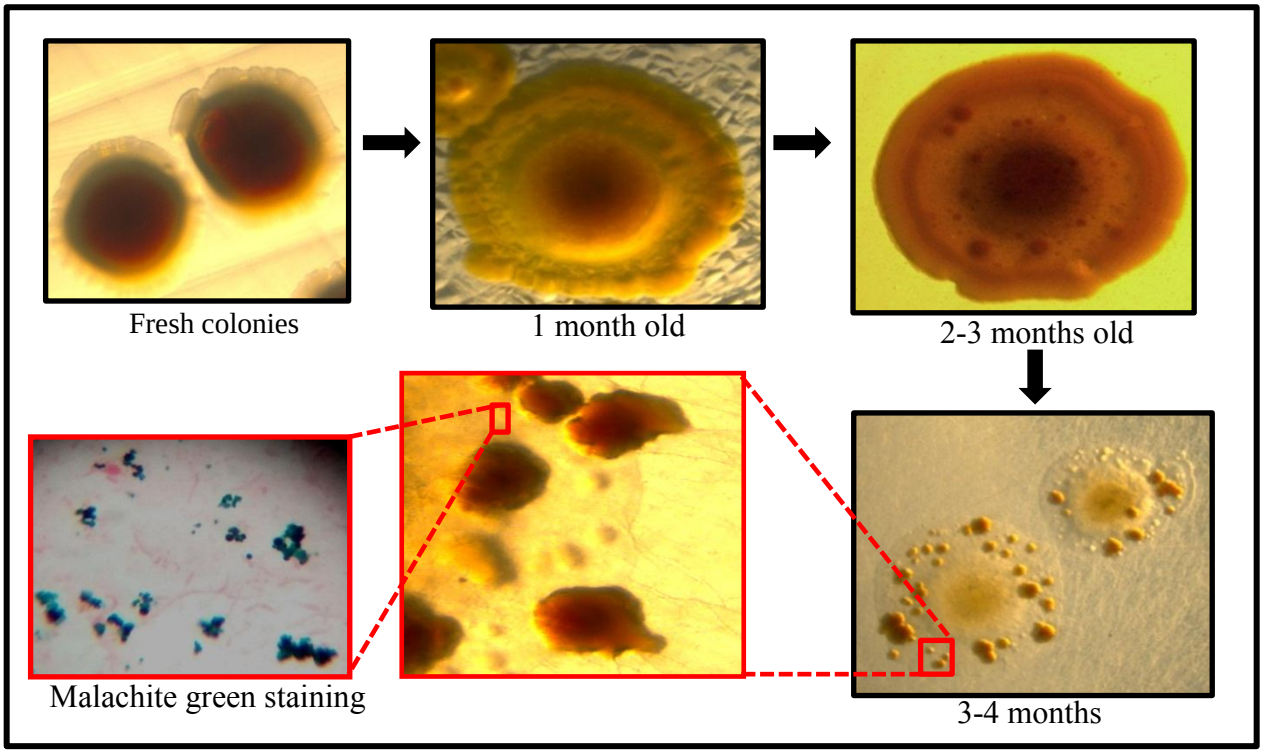


Figure 3.12 Microscopic images for microcolonies formation in *Rba. johrii* colonies.

3.1.4 Endospores were also identified in *Rhodobacter alkalitolerans* JA916

This strain is isolated from alkaline brown pond in adverse condition culture was grown at 30°C on modified mineral medium, in the screw capped tubes in 30°C incubation and 2400lx light intensity. 1 week old culture was checked for spores by phase contrast and malachite green and Nile blue staining (Fig. 3.13) microscopy suggest presence of endospore.

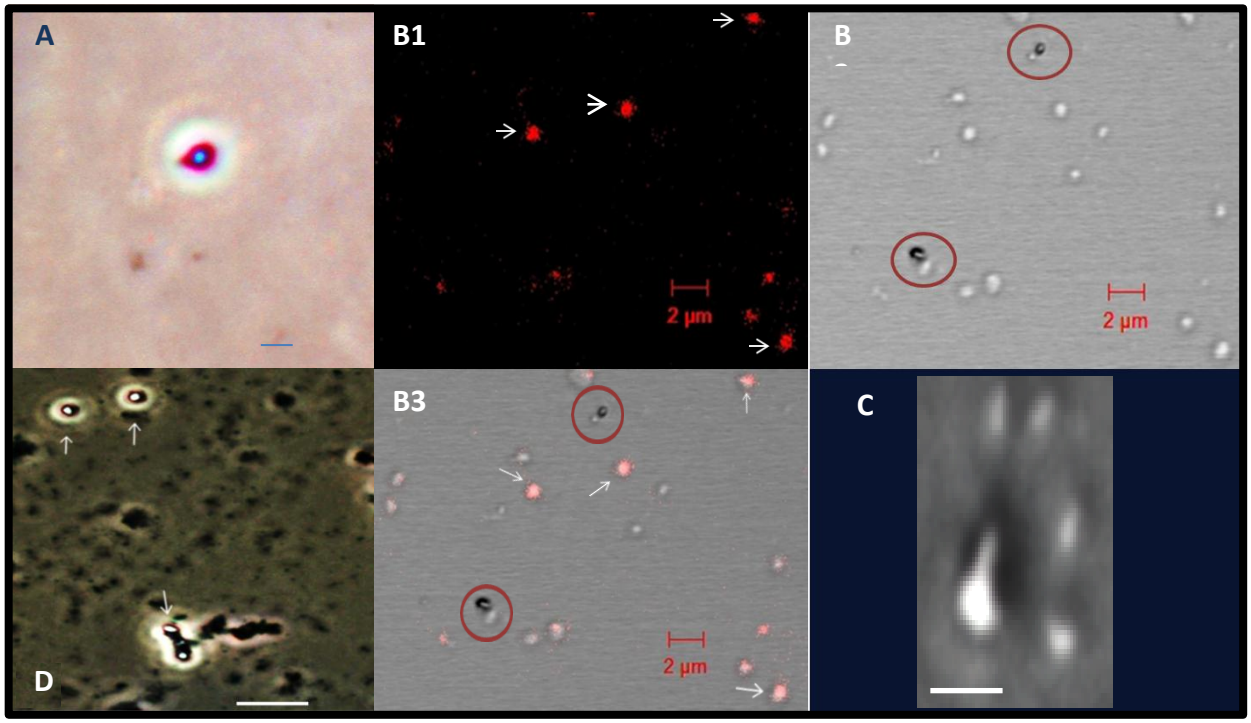


Figure 3.13 Endospores identified in *Rba. alkalitolerans* JA916 (A) Schaeffer and fulton staining using malachite green and saffranin (B1) Fluorescent staining with nile red (B2) Bright field microscopy, red circle indicate spores (B3) Merge image here white arrow represent Pha granule stained with nile red, red circle is spores (C) Phase contrast image of spore germination (D) Phase contrast image of refractile spores.

3.2 Genomic information and mining for sporulation gene homologs in *Rba. johrii*

The objective was to identify the homologs of sporulation genes from the genome of *Rba. johrii*. To address this question, the genome of *Rba. johrii* was sequenced and mined for the spore genes using *Bacillus subtilis* known spore genes as reference. In addition, the genome of *Rba. johrii* was characterized in detail and compared with the other valid species names of the genus *Rhodobacter*.

3.2.1 Genome characterization and comparison

3.2.1.1 Genomic DNA isolation

Rba. johrii genomic DNA was extracted using the kit. The quality of isolated DNA was checked on the Agarose gel, DNA was not sheared and intense band appeared on the gel (Fig. 3.2.1)

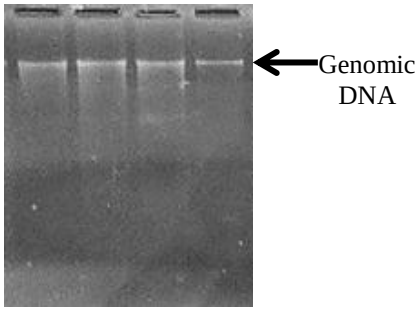


Figure 3.2.1 Isolated genomic DNA of *Rba. johrii* on agarose gel.

3.2.1.2 Quantification of isolated DNA

DNA concentration was 302 ng/µl when quantified. The purity of DNA was measured at $A_{260/280}$ Ratio to be 1.73 which is less than 1.8 therefore the DNA was pure and free of RNA contamination (Table 3.2.1).

Table 3.2.1 Genomic DNA quantification

Organism name	Qubit Conc.(ng/µl)	Nanodrop Conc.(ng/µl)	$A_{260/280}$ Ratio
Rhodobacter johrii	200	302.3	1.73

3.2.1.3 Assembly and annotation

Sequencing was carried out and resulted in 2,959,826 raw reads. After de novo assembly, the CISA integrated and generated 257 contigs with total genome length of 4,512,111 base pair respectively.

3.2.1.4 Genome characteristics

Genome size of *Rba. johrii* was 4,512,111 bp with 69.1 mol% G+C content. Total numbers of genes were 4333, total CDS regions were 4269, protein encoding genes (PEG) were 4035, rRNA genes were 64, tRNA coding genes were 53, ncRNAs genes were 3 and pseudo genes were 234, hypothetical protein encoding genes were 1075 predicted in genome sequence of *Rba. johrii* (Table 3.2.2). The genomic DNA of *Rba. johrii* was pure as confirmed from the genome sequencing which had no contaminating sequences of other taxa. The scaffolds sequences of the draft assembly have been deposited at NCBI GenBank (Bio Project number "PRJNA323784") with accession number as MABH00000000.

Table 3.2.2 Genome information and characteristics of *Rba. johrii.*

Sequencing platform	Illumina HiSeq
Sequencing Depth	150.0x
Assembly Method	MaSuRCA v. 2.2.1; SPAdes v. 3.1.1
Contigs	257
Genome ID	445629.4
GenBank Accessions	MABH00000000
Genome Size	4,512,111 bp
G+C content	69.1 %
Genes (total)	4,333
CDS (total)	4,269
PEG (protein encoding gene)	4,035
RNA genes	64
TRNAs	53
NcRNAs	3
Pseudo Genes (total)	234
hypothetical protein encoding genes	1,075

3.2.1.5 Gene Ontology: GO term for predicted CDSs or subsystem distribution of genes

The coding gene sequences were categorized according to the function of genes which was categorized into three processes. Biological process involving 107 genes, molecular function involving 175 genes and cellular components involving 51 genes (Table 3.2.3). The number of genes involve in biological processes is divided further into the sub-system (Fig. 3.2.2) similar way the molecular function (Fig. 3.2.3) and cellular components (Fig. 3.2.4) were also divided.

Table 3.2.3 Gene Ontology terms identified in each category.

Category	Number of terms
Biological Processes	107
Molecular Functions	175
Cellular Components	51

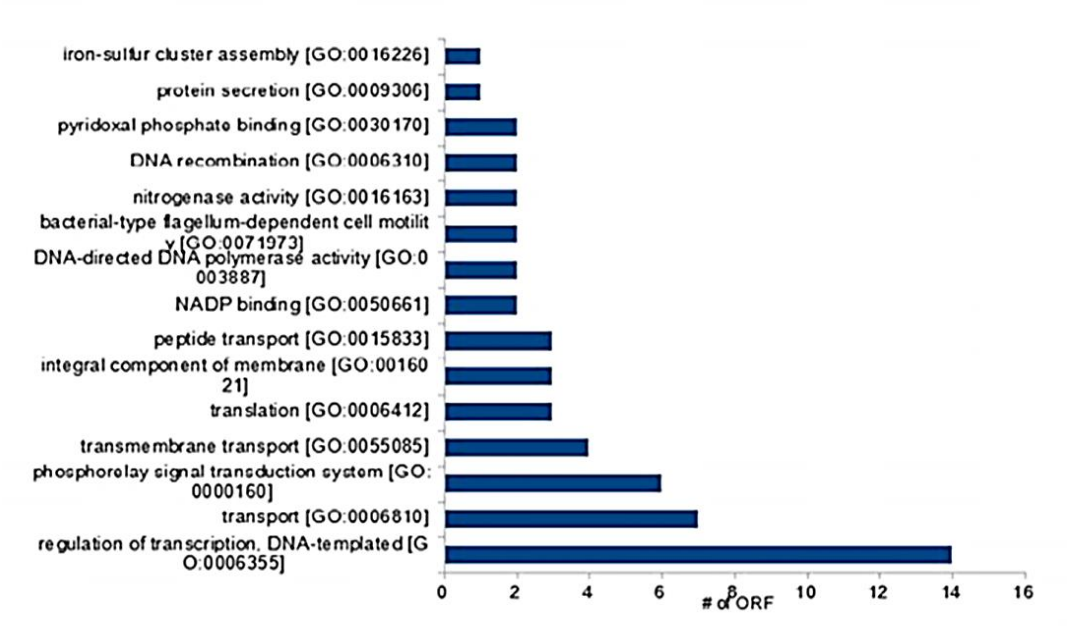


Figure 3.2.2 Top 15 terms in biological function category from GO annotation.

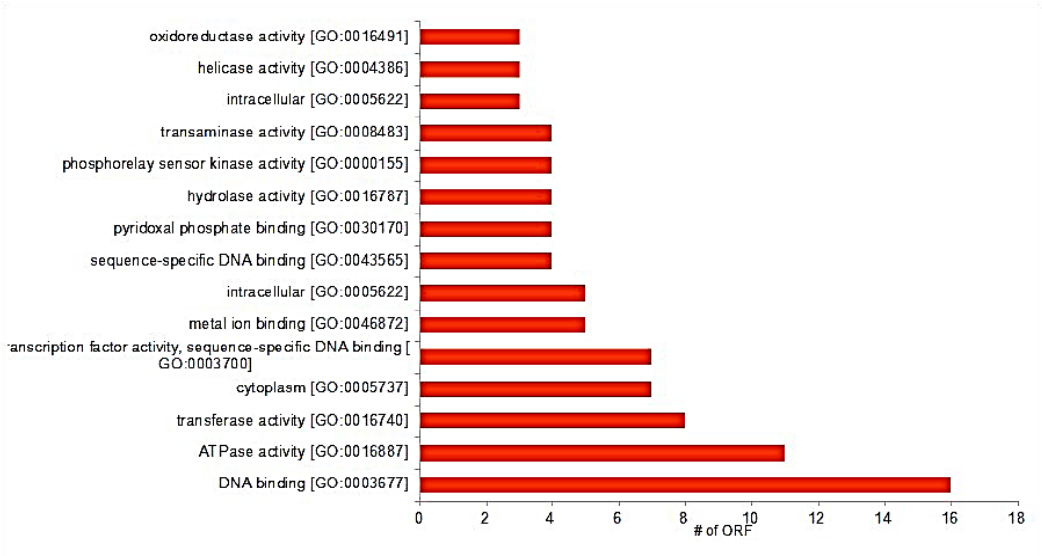


Figure 3.2.3 Top 15 terms in molecular function category from GO annotation.

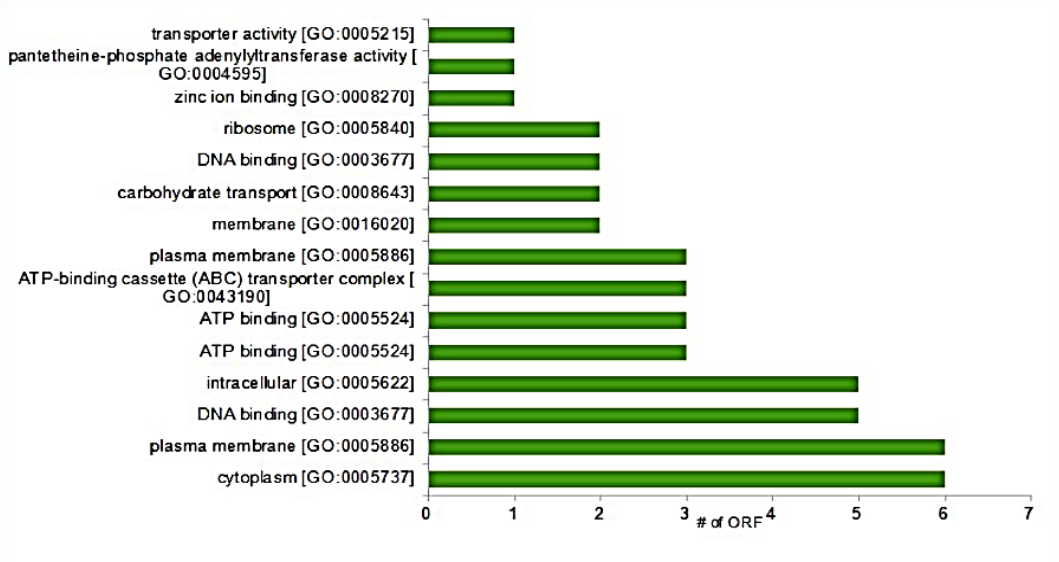


Figure 3.2.4 Top 15 terms in cellular component category from GO annotation.

3.2.1.6 Circular genome map

Rba. johrii genome sequence was represented in a circular form which showed GC skew, GC content, drug targets, transporters, virulence factors, antimicrobial resistance genes, Non CDS, CDS reverse., CDS forward, and contigs (Fig. 3.2.5).

3.2.1.7 Analysis of secondary metabolite biosynthesis gene clusters

Rba. johrii genome has diverse gene clusters for various secondary metabolites which includes terpene, hserlactone, and carotenoid producing gene clusters (Fig. 3.2.6). Secondary metabolite genes analysis is done by AntiSMASH (Table 3.2.4) showed involvement of total 84 genes.

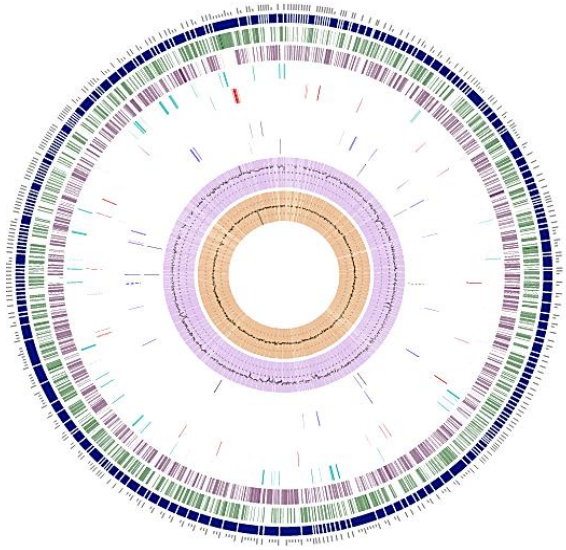


Figure 3.2.5 The rings from the inner to outer circle are, GC skew, GC content, Drug targets , transporters ,virulence factors, antimicrobial resistance genes ,Non CDS, CDS rev. , CDS for. , contigs.

Table 3.2.4 Secondary metabolite biosynthetic gene cluster and genes from AntiSMASH

Strain name	No. of clusters	Clusters name	Total genes	Core biosynthetic genes	Additional biosynthetic genes
Rhodobacter johrii	C-1	Terpene	10	1	2
	C-2	Terpene	14	1	5
	C-3	Hserlactone	18	2	0
	C-4	Terpene	10	1	2
	C-5	Terpene	14	1	5
	C-6	Hserlactone	18	2	0
TOTAL	**6**		**84**	**8**	**14**

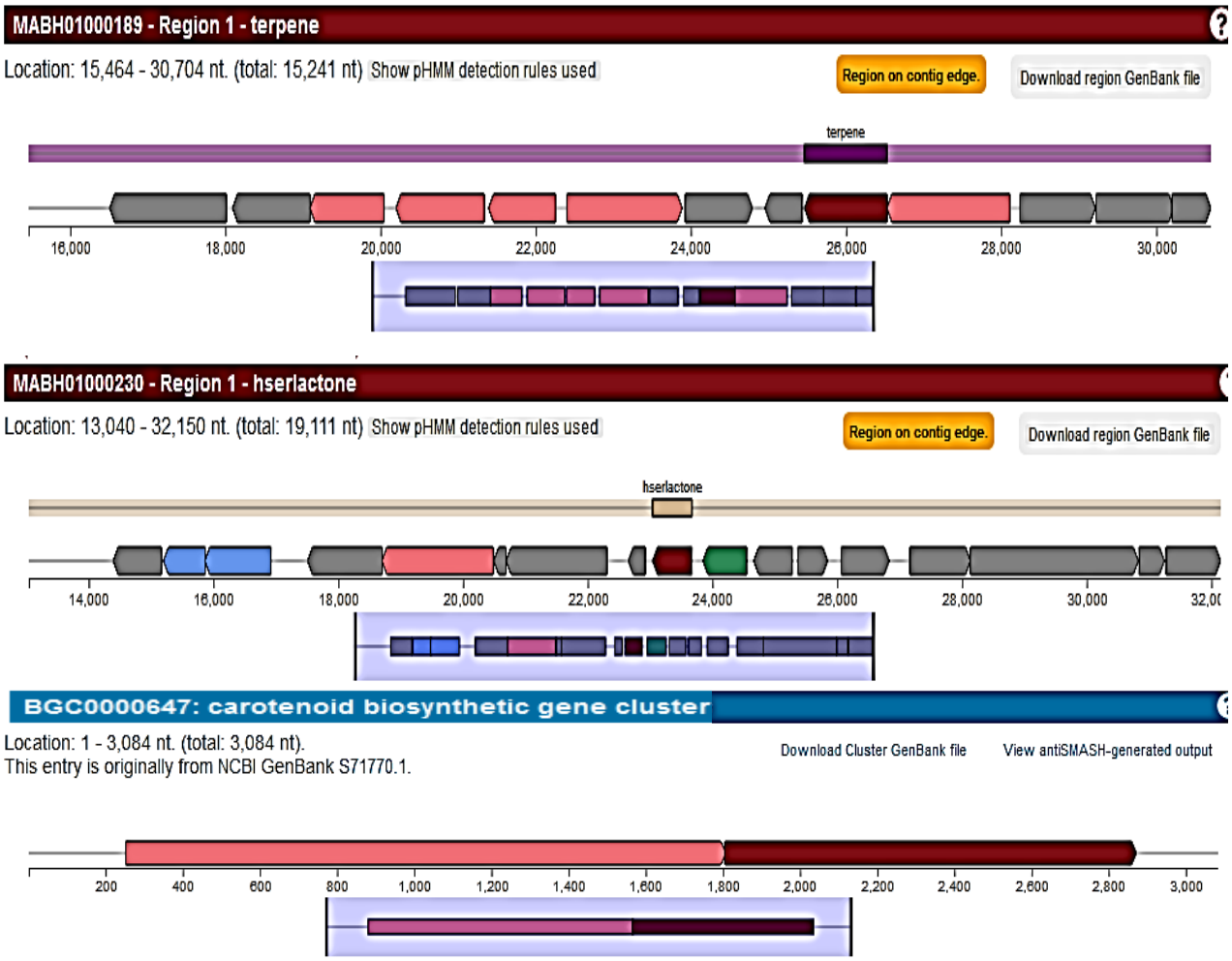


Figure 3.2.6 Secondary metabolite biosynthesis gene clusters.

3.2.1.8 CRISPR- CAS system analysis

CRISPR- CAS system is defense mechanism against invading bacteriophage DNA or plasmid DNA. A total 11 CRISPR loci were detected in *Rba. johrii* (Table 3.2.5). Comparative CRISPR-CAS loci analysis was done with all the Rhodobacter genus members present at the time of analysis, among them *Rba. johrii* was the organism having highest number of CRISPR-CAS loci (Fig. 3.2.7).

Table 3.2.5 Eleven CRISPR locus and their property

Crisper id	Crisper Length	DR consensus	DR length	No. of spacers
tmp_30	105	5CTGTCCGGCGCTGCCGCCGGGCAGGC3	26	1
tmp_35	129	5TGGCAGCCCCCGCGCGGGGCGCCTCCCCTTGACGATGCGTGC3	42	1
tmp_53	78	5GGTTTGTTTCGCGCGCGAAACAA3	23	1
tmp_63	106	5GCCGAGCGGGGCCGGACCTCCCGA3	24	1
tmp_125	149	5CGTGGCCCGGGTGGAAGGTCCGGGGGCGTCGGTAAGGGGTGGGGA3	45	1
tmp_136	105	5AGTGTCCGAAATCCGGACACGGC3	23	2
tmp_160	131	5CGTCGGACGATCCGCGCGTGCGACGTCAGGCCGGCCGC3	38	1
tmp_163	96	5GAGAGCAGGCGACCCTCACGCCTGCC3	26	1
tmp_191	86	5GTGCCGCCCGCGCGTGAGAGGATCCG3	26	1
tmp_242	110	5GGGGAGATCGGGCGGCGGCTCGGGGCCG3	28	1
tmp_248	203	5CAAGGCGCCACGCGCGCCGGCAGCGGA3	27	3

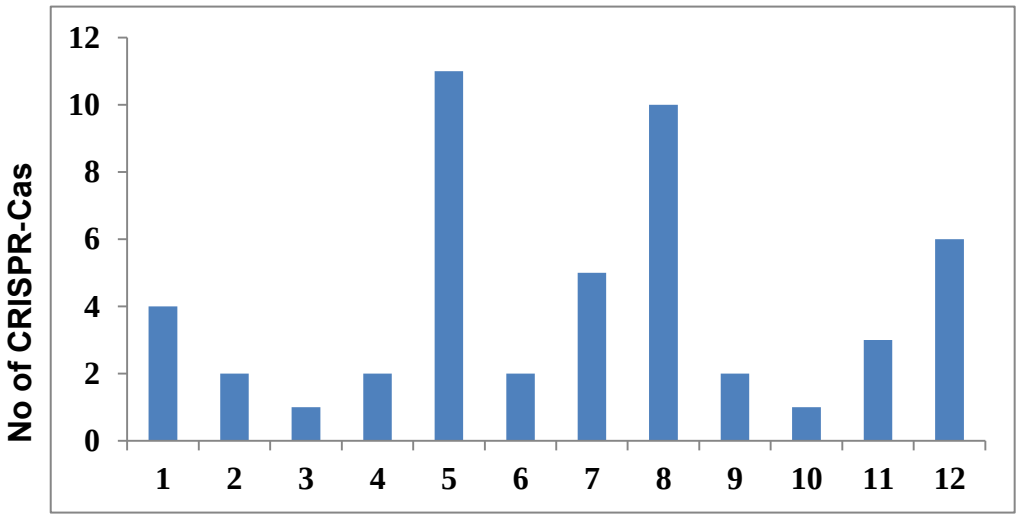


Figure 3.2.7 Comparative analysis of CRISPR-CAS Loci of *Rhodobacter* members.1- *Rhodobacter aestuarii* JA296, 2- *Rhodobacter azotoformans* KA25,3- *Rhodobacter blasticus* DSM2131, 4- *Rhodobacter capsulatus* ATCC 11166, 5- *Rhodobacter johrii* JA192, 6- *Rhodobacter maris* JA276, 7- *Rhodobacter megalophilus* JA194, 8- *Rhodobacter ovatus* JA234 9- *Rhodobacter sphaeroides* 2.4.1, 10- *Rhodobacter veldkampii* ATCC 35703, 11- *Rhodobacter vinaykumarii* JA123, 12- *Rhodobacter viridis* JA737

3.2.1.9 CAZymes (Carbohydrate-Active Enzymes) analysis

Here the protein families of glycosyltransferases, glycoside hydrolases, polysaccharide lyases, non-catalytic carbohydrate-binding module enzymes and carbohydrate esterases which take part in metabolism, synthesis were detected. *Rba. johrii* has 510 genes associated with the carbohydrate metabolism, which is the highest among the Rhodobacter members and gene clusters CGC34 and CGC35 are only present in *Rba. johrii* compare to other Rhodobacter spp.

3.2.2 Genome mining of *Rba. johrii* for homologs of major key spore proteins according to stage wise of canonical sporulation pathway

BLASTp analysis was performed to find the common spore proteins *Rba. johrii* and Firmicutes. *B. subtilis* subsp. *subtilis* 168 genome (Accession No. NC000964) as query was BLAST against the *Rba. johrii* complete proteins. Around one hundred major protein homologs were detected in *Rba. johrii*, while those sporulation proteins which also have functions in normal cell-functioning (other than spore-formation) were not considered for analysis. To find the similarities and differences in the sporulation cycle in both the system, we searched the protein homologs according to the stages of sporulation.

3.2.2.1 Homologs of stage I (Starvation state)

In the first stage of sporulation, the cell detects environmental stress which stimulates spore formation via signaling cascade involving σH, σA, KinA - KinE, Spo0F and Spo0B. Spo0A is a key protein which regulates many genes in sporulation. Except Spo0B, all the other protein homologs were detected in *Rba. johrii* with 28-68% amino acid identity and protein coverage of 21-92% (Table 3.2.6).

Table 3.2.6 Major proteins participating in Starvation state – activation of Spo0A state in *B. Subtilis* and identification of their homolog proteins in *Rba. johrii*.

***B. subtilis* protein**	***Rba. johrii* protein**	**Accession no. of *Rba. johrii* protein**	**Identity (%)**	**Coverage (%)**
Kin A	response regulator	WP084295807	34	39
Kin B	PAS domain-containing protein	WP069330358	28	57
Kin C	PAS domain - containing protein	WP069333518	29	52
Kin D	sensor histidine kinase	WP069331412	31	41
Kin E	response regulator	WP101327650	30	43
Spo0F	response regulator	WP002720740	33	92
Spo0A	response regulator transcription factor	WP069333547	36	43

3.2.2.2 Homologs of stage II (Onset of sporulation)

In this stage, the cell is asymmetrically divided by formation of septum. The proteins involved in this process are σ^H, σ^E, FtsZ, SpoIIE, Spo0A, RacA, SpoIIM, SpoIIP, and SpoIID. All of these protein homologs except RacA and SpoIIP are present in *Rba. johrii*. The identity of these protein homologs ranged from 19-51% with 17-95% protein coverage (Table 3.2.7).

Table 3.2.7 Major proteins participating in onset of sporulation in *B. subtilis* and identification of their homolog proteins in *Rba. johrii*.

***B. subtilis* Protein**	***Rba. johrii* protein**	**Accession no. of *Rba. johrii* protein**	**Identity (%)**	**Coverage (%)**
Spo0A	response regulator transcription factor	WP069333547	36	43
FtsZ	cell division protein FtsZ	WP069331225	51	83
SpoIIE	fused response regulator/phosphatase	WP108223412	24	17
SpoIID	DUF3035 domain-containing protein	WP069330774	33	17
SpoIIM	MlaE family lipid ABC transporter permease subunit	WP069331900	29	29

3.2.2.3 Homologs of stage III (Commitment to sporulation and engulfment)

In this stage, the cell's genetic material is also partitioned and transported to the forespore with the help of key protein SpoIIIE. Other proteins involved in this stage of sporogenesis are

SpoIIID, SpoIIE, GerR, SpoIIQ, SpoIVFA, SpoIIAA, SpoIIAB, SpoIIGA, σE, σF, σA, σG SpoIIR, Gin, RsfA of these homologs SpoIIGA, SpoIIR SpoIIID, GerR and Gin are absent in *Rba. johrii*. The identity of protein homologs ranged between 22-50% with a protein coverage of 16-95% (Table 3.2.8).

3.2.2.4 Homologs of stage IV (Completion of engulfment and cortex synthesis)

The characteristic endospore within the cell is finally formed and visible in this stage. Many structural proteins that form the spore coat and cortex layer are produced. Key proteins in this stage are SpoVIF, SpoVT, GerE, GerR, SpoIVB, Gin, SpoIIQ-SpoIIIAH-G-K channel, SpoIIQ, RsfA, σG (spoIIIG, sigG), σK (spoIIIC, spoIVCB), SpoIIID, and SleB. Among these protein orthologs SpoVIF, SpoIIQ-SpoIIIAH channel and SpoVT were not detected in *Rba. johrii*. The identity of protein orthologs ranged between 31-41% having 11-72% protein coverage (Table 3.2.9).

Table 3.2.8 Major proteins participating in sporulation and engulfment (Stages II-III) in *B. subtilis* and identification of their matching proteins in *Rba. johrii*.

***B. subtilis* protein**	***Rba. johrii* protein**	**Accession no. of *Rba. johrii* protein**	**Identity (%)**	**Coverage (%)**
SpoIIQ	peptidoglycan DD-metallo endopeptidase family protein	WP069332504	35	35
SpoIIE	4-hydroxy-4-methyl-2-oxoglutarate aldolase	WP069331967	27	34
SpoIVFA	ABC transporter ATP-binding protein	WP069331875	27	28
Spo0A	response regulator transcription factor	WP069333547	36	43
SpoIIAA	STAS domain-containing protein	WP112330558	22	93
SpoIIAB	sensor histidine kinase	WP069332910	39	30
RsfA	hypothetical protein	WP069333566	50	16
spoIIIE	cell division protein FtsK	WP108223489	45	60

Table 3.2.9 Major proteins participating in completion of engulfment and cortex synthesis in *B. subtilis* and identification of their matching proteins in *Rba. johrii*.

***B. subtilis* protein**	***Rba. johrii* protein**	**Accession no. of *Rba. johrii* protein**	**Identity (%)**	**Coverage (%)**
GerE	DNA-binding response regulator	WP009562094	41	72
SpoIVB	Do family serine endopeptidase	WP069333596	31	11
SpoIIQ	peptidoglycan DD- metallo endopeptidase family protein	WP069332504	35	35
RsfA	hypothetical protein	WP069333566	50	16
SleB	cell wall hydrolase	WP069331589	33	43

3.2.2.5 Homologs of stage V-VI (Coat synthesis and spore maturation)

By this stage, there is development of cortex layer resulting in the formation of thick protective layers. Major proteins involved here are coat proteins CotB, CotW, CotQ, CotG, CotS, YlbD, YtxO, LipC, YeeK, CotU, CotD, YxeE, CotE, YhaX, SpoVM, SpoIVA, SpoVID, YuzC, YaaH, YncD, YheD, CotO, CotM, YhjR, YknT, YsxE, CwlJ, YutH, Tgl, YisY, CotZ, YybI, CotT, CotA, CotP, YsnD, and OxdD. In *Rba. johrii* CotG, YeeK, YlbD, LipC, YxeE, CotU, CotD, CotE, SpoVM, SpoVID, YaaH, Yknt, YuzC and YsxE proteins were missing. The identity of other protein homologs ranged between 26-55% with 7-97% protein coverage (Table 3.2.10).

Table 3.2.10 Major proteins participating in coat synthesis and spore maturation in *B. subtilis* and identification of their matching proteins in *Rba. johrii*.

***B. subtilis* protein**	***Rba. johrii* protein**	**Accession no. of *Rba. johrii* protein**	**Identity (%)**	**Coverage (%)**
CotB	Hypothetical protein	WP069333635	55	12
CotW	DUF1150 domain-containing protein	WP069333415	33	57
CotQ	trimethylamine methyltransferase	WP069331896	29	7
CotS	endonuclease/exonuclease/phosphatase family protein	WP069331690	27	16
CotA	hypothetical protein	WP069332063	33	27
CotJC	hypothetical protein	WP069332008	63	23
CotO	AAA+ family ATPase	WP069331348	45	22
CotSA	hypothetical protein	WP069331767	24	57
CotM	hypothetical protein	WP146757676	30	33
CotZ	ATPase	WP069333252	39	24
SpoIVA	hypothetical protein	WP069331425	33	42
YncD	alanine racemase	WP069333085	29	97
YheD	DUF3576 domain-containing protein	WP069330480	29	42
YhjR	phage major tail protein, TP901-1 family	WP009564481	36	20
YutH	hypothetical protein	WP084295997	26	82

3.2.2.6 Homologs of stage VII (mother cell lysis and germination)

In this final stage mother cell is lysed, spore is expelled and germinates during favorable conditions. Many proteins play a role in these stages including GerF, GerA family, GerB family, GerM, YpeB, GerC family, YtgP, GerK, NucB, GerD, SpoIIIC, SpoIVCA and SpoIVCB. From these many homologs GerF, YpeB, YtgP, GerB, GerC, GerM, GerD, GerK, SpoIVCA, SpoIIIC, NucB, PrkC and SpoIVCB are absent in *Rba. johrii* (Table 3.2.11).

Table 3.2.11 Major proteins participating in mother cell lysis (Stage VII) and spore germination and outgrowth in *B. subtilis* and identification of their matching proteins in *Rba. johrii*.

***B. subtilis* protein**	***Rba. johrii* protein**	**Accession no. of *Rba. johrii* protein**	**Identity (%)**	**Coverage (%)**
GerA	DUF1049 domain-containing protein	WP069331564	33	61
GerE	DNA-binding response regulator	WP009562094	41	72
GerQ	NADH-quinone oxidoreductase subunit A	WP002719664	35	24
GerPE	DUF1737 domain-containing protein	WP069332810	27	76
GerPC	cell division protein ZapA	WP069332829	32	20
CwlH	murein L,D-transpeptidase	WP084295534	30	30
CwlC	N-acetylmuramoyl-L-alanine amidase	WP069332500	34	30
SpoVAD	Hypothetical protein	WP069332049	27	68

3.2.2.7 Homologs of major sporulation-specific sigma factors

All major sporulation-specific sigma factors of *B. subtilis* i.e., σ^H, σ^A, σ^E, σ^F, σ^G , σ^K show similarity to RNA polymerase sigma factors, DNA binding proteins and other proteins of *Rba. johrii* (Table 3.2.12).

Table 3.2.12 Major RNA polymerase sigma factor participating in all the stages of sporulation in *B. subtilis* and identification of their matching proteins in *Rba. johrii*.

***B. subtilis* protein**	***Rba. johrii* protein**	**Accession no. of *Rba. johrii* protein**	**Identity (%)**	**Coverage (%)**
σ^H	helix-turn-helix domain-containing protein	WP069332496	33	21
σ^A	sigma-70 family RNA polymerase sigma factor	WP112330062	40	20
σ^E	RNA polymerase sigma factor RpoH	WP069332369	36	38
σ^F	RNA polymerase sigma factor RpoD	WP069332997	29	95
σ^G	paraquat-inducible protein A	WP002719611	31	23
σ^K	RNA polymerase sigma factor RpoD	WP069332997	27	82

Other proteins involved in sporulation are shown in (Table 3.2.13) with identity range of 20-48% and coverage range of 11-82%. However, some of the spore proteins like Small Acid Soluble Proteins (SASPs) and DPA synthetase are absent in *Rba. johrii*. All these putative proteins showing important spore-related functions have been mapped onto a circular representation of *Rba. johrii* genome (Fig.3.2.8). From the pool of putative proteins of sporogenesis in *Rba. johrii* a Spore Cortex Lytic Enzyme (Sle) protein homolog has been characterized in this study from *Rba. johrii*.

Table 3.2.13 other proteins participating in sporulation in *B. subtilis* and identification of their matching proteins in *Rba. johrii.*

B. subtilis protein	Identity (%)	Coverage (%)	*B. subtilis* protein	Identity (%)	Coverage (%)
YtaF	36	21	**PbpF**	37	66
SkfB	26	82	**SplB**	20	44
Gdh	36	80	**SpoVE**	31	82
YdhD	28	43	**OxdD**	25	65
YfnH	28	28	**SqhC**	28	51
YhcV	26	31	**CgeD**	37	38
YhaL	30	11	**CgeB**	26	28
SpoVB	30	24	**IonB**	28	52
SpoVK	44	79	**RefZ**	25	36
SpoVAF	48	15	**KatX**	23	70
SpoVAD	27	68	**TepJ**	42	17
SpoVAB	33	37	**SpoVG**	44	25
SpoVAA	31	34	**YisJ**	33	16
Yqfc	34	35	**AsnO**	36	15
YtvI	33	23	**SkiZ**	24	59
IytH	30	19	**RicF**	23	38
SpoIID	33	17	**YjqC**	46	89
Pbpg	30	76	**KbaA**	31	22
HtrC	24	68	**SpaC**	30	17
CwlC	34	30	**YkvP**	20	49
CwlH	30	30	**YkvT**	35	45

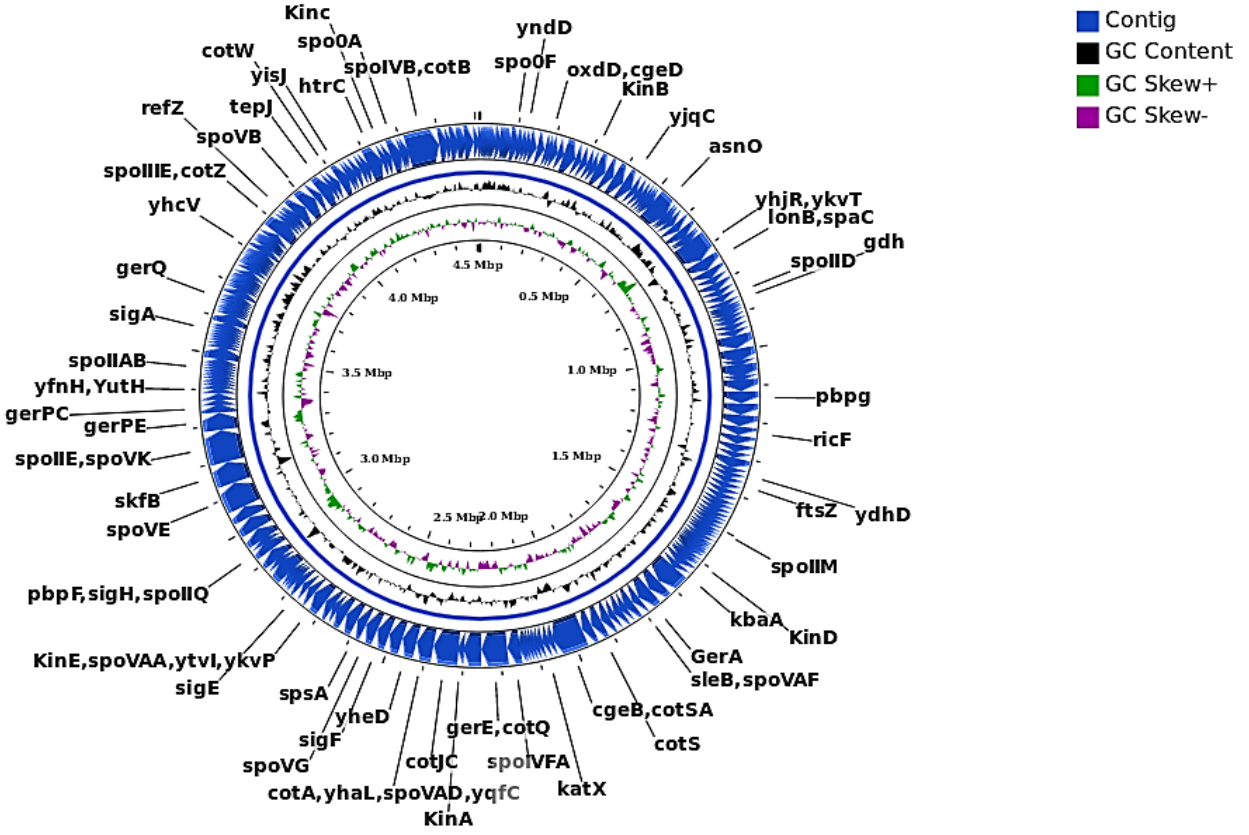


Figure 3.2.8 Mapping of homologs of endospore-formation genes in genome of *Rba. johrii.* Blue colored circle represents the contigs, black circle shows GC content, green circle shows GC Skew + and purple color shows GC Skew.

3.3 *In silico,* biochemical and functional characterization of a putative spore cortex lytic enzyme (SleR) protein of *Rba. johrii*

Identification of 87 major sporulation associated protein homologs of *B. subtilis* were identified in *Rba. johrii* (chapter 3.2 of thesis) with identities ranging from 19-68%. From this pool, a novel putative spore cortex lytic enzyme (Sle) protein was identified with protein identity of 28.4% to spore cortex lytic enzyme (Sleb) of *Bacillus subtilis* subsp. *subtilis*. Sleb is an important and well-characterized protein in spores of *B. subtilis* (Boland *et al*. 2000). This homolog was characterized in *Rba. johrii* using *in silico, in vitro* approaches and *in vivo* transcriptional profiling to find out its association with endospore like refractile bodies of *Rba. johrii*.

3.3.1 *In silico* characterization of putative sporulation protein spore cortex lytic enzyme of *Rba. johrii*

We have identified a protein annotated as cell wall hydrolase to be a putative spore cortex lytic enzyme (Sle) (Accession no. WP069331589) in *Rba. johrii* which is 240 amino acid in length. This protein is having 28.4% amino acid identity and 67% protein coverage to the *Bacillus subtilis* subsp. *subtilis* and 26.3% identity, 65% coverage with *B. cereus* ATCC 14579 spore cortex lytic enzyme (SleB) protein. BLAST analysis of full length Sle of *Rba. johrii* protein sequence with spore cortex-lytic enzyme (*Bacillus anthracis*) showed 27% sequence identity and 65% query cover and only of catalytic domain sequence showed 28% sequence identity and 82% query cover.

3.3.1.1. Characteristics of the spore cortex lytic (*Sle*) gene of *Rba. johrii*

The promoter region was identified for the putative *sle* gene of *Rba. johrii* (Fig. 3.3.1) which has -10 box (CGCAAAAAT) from 121 bp to 113 bp and -35 box (TGGACG) from 141 bp to 136 bp upstream of the start codon (ATG) of coding sequence having total length 723 bp of

the *sle* gene. 240 amino acid polypeptide is encoded by the *Sle* gene expected to have molecular weight of ~25,557 Da, which is highly basic having a theoretical pI of 9.82.

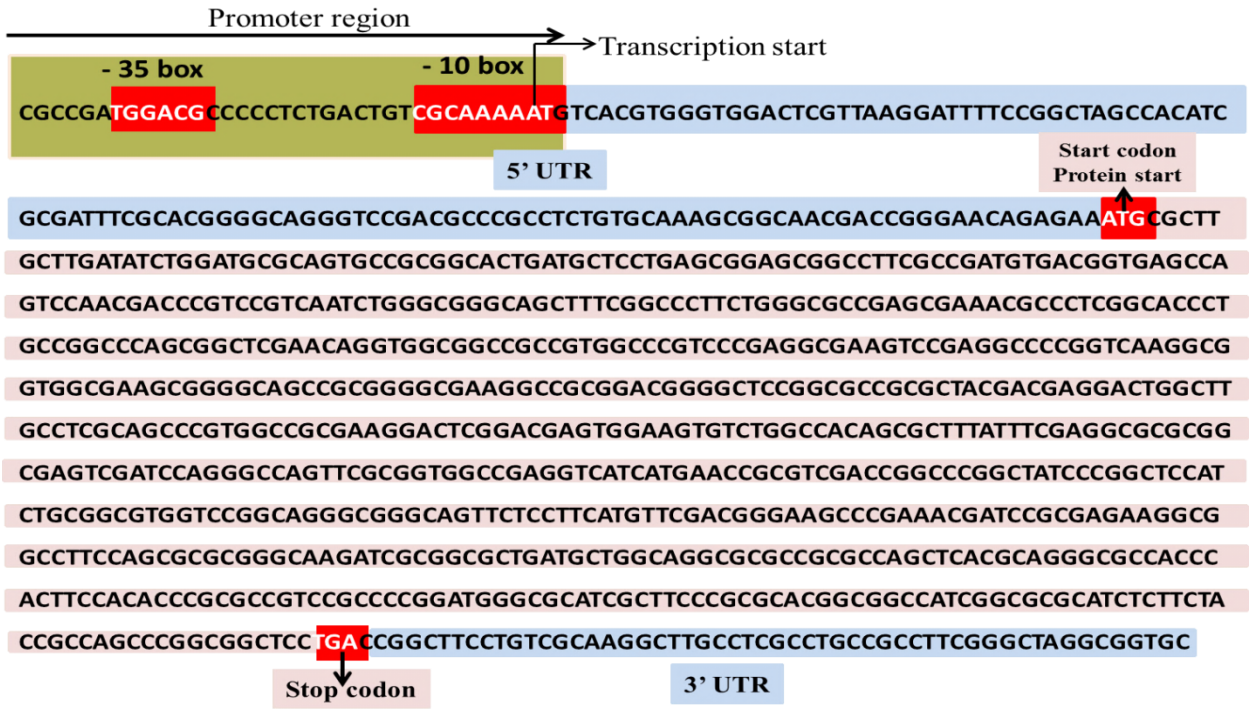


Figure 3.3.1 Promoter and gene properties of *Sler* from *Rba. johrii*. Promoter region (grey region) consist of -10 box and -35 box (red region) transcription start site transcribes the 5' untranslated (5'UTR) region (Blue region at 5') from start codon (ATG) protein is coded till the stop codon (TGA) (Pink region) after which is the 3' untranslated region (3'UTR) (Blue region at 3').

3.3.1.2. Sequence similarity, protein domain prediction and its conserved sequences

Protein characteristics and functional domains was done with the SMART search tool (Table 3.3.1) which had predicted signal peptide from 1 to 23 a.a and a conserved hydrolase 2 super family (Pfam 07486) domain from 131 to 234 a.a (Fig. 3.3.2). Multiple sequence alignment of full-length Sle protein of *Rba. johrii* with Sleb protein of *Bacillus* spp. showed the conserved domain of the catalytic site with divergence at other parts in the protein. The signature sequence motif EXRGE (Jing *et al.*, 2012) the alternate glutamate (E) was conserved at the

position Glu128 and Glu132, simultaneously the catalytic residue glutamate (Glu128) residue was also conserved in Sle of *Rba. johrii* (Fig. 3.3.3).

Table 3.3.1 Characteristics of SleR protein of *Rba. johrii* analysed by SMART server predicting domains, repeats, motifs and features.

Protein	Pfam Domain	Pfam domain accession	Position of domain	E-value:	GO function	Signal peptide	Low complexity region	Homology with known structure PDB domain ID
SleR	Hydrolase_2	PF07486	131 to 234 a.a	3e-25 (HMMER3)	hydrolase activity (GO:0016787)	1 to 23 a.a	Not present	4FET|A, 4f55.1

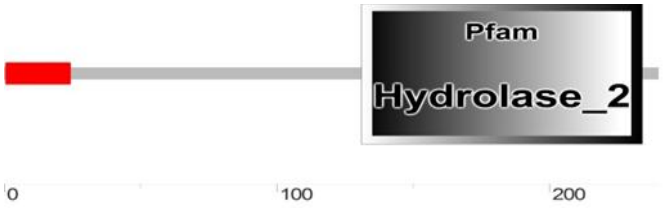


Figure 3.3.2 Detection of conserved domain using Simple Modular Architecture Research Tool (SMART), red part indicate the signal sequence, Box represent hydrolase catalytic domain.

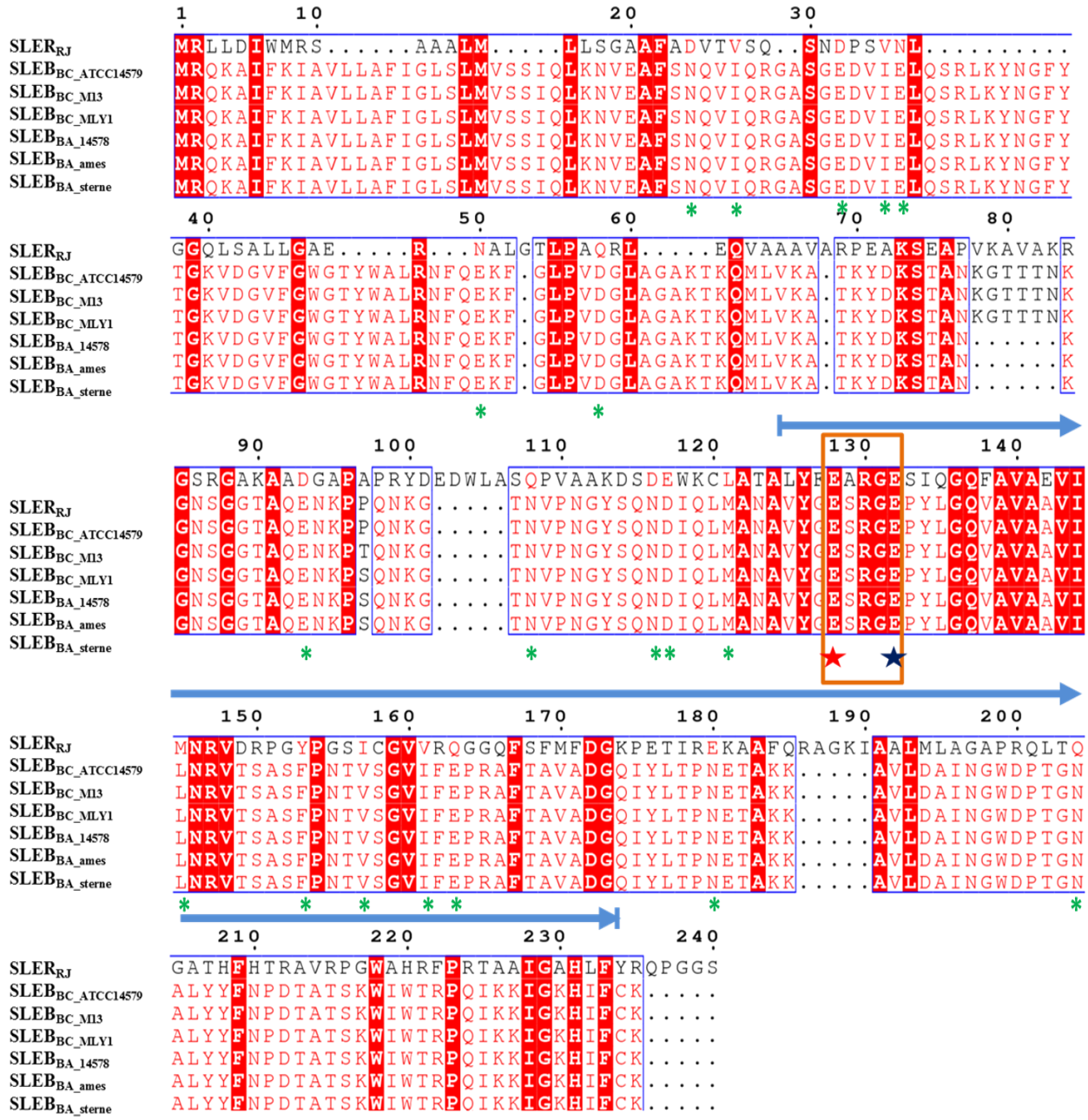


Figure 3.3.3 Multiple sequence alignment of full length SleR protein from *Rba. johrii* with SleB protein of *B. cereus* ATCC14579 ($SLEB_{BC\_ATCC14579}$), *B. cereus* M13 ($SLEB_{BC\_M13}$), *B. cereus* MLY1 ($SLEB_{BC\_MLY1}$), *B. anthracis* ATCC14578 ($SLEB_{BA\_14578}$), *B. anthracis* Sterne ($SLEB_{BA\_sterne}$), *B. anthracis* Ames ($SLEB_{BA\_ames}$). Conserved amino acids are shown in red boxes, green asterisk mark represents the similar residues. The catalytic residue Glu is highlighted with red star mark and another Glu with blue star mark these alternate sequences are part of SleB specific EXRGE motif shown in an orange box. Blue line donates the catalytic domain of the proteins.

3.3.1.3 3D secondary structure prediction and its validation

The full length secondary structure of Sle was predicted by using the RaptorX server (Fig. 3.3.4A) and SWISSMODEL server based on homology modeling the template used for modeling was taken from the germination-specific lytic transglycosylase SleB from *Bacillus cereus* ATCC 339 14579 (PDB ID - 4f55.1; Li *et al*., 2012). Therefore according to this template the model drawn from 109 to 235 a.a, which include hydrolase domain. Structure has seven α-helices, three β-sheets, eleven loops and turns (Fig. 3.3.4B). Model drawn was validated using PROCHECK tool, proteins ramachandran plot indicating >99% residues in allowed region and only one residue amounting to ~1% was in disallowed region (Fig. 3.3.5B). Sle model of *Rba. johrii* and its template model was superimposed based on Cα position using PyMol. The root mean square deviation (RMSD) for the superimposition of model and template was found to be 1.96, which is less than 2.0 hence model is validly accepted (Fig. 3.3.5A).

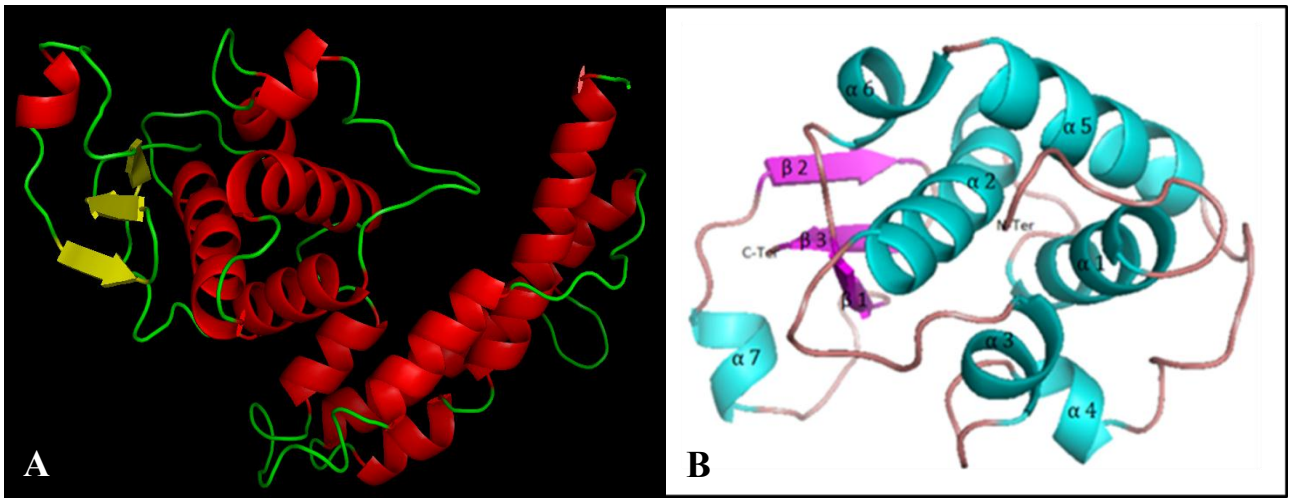


Figure 3.3.4 (A) Model generated with help of RaptorX (B) Homology model of SleR constructed using SWISSMODEL α helices are in cyan colour, β- sheets are in pink colour and other colors represent loops and turn.

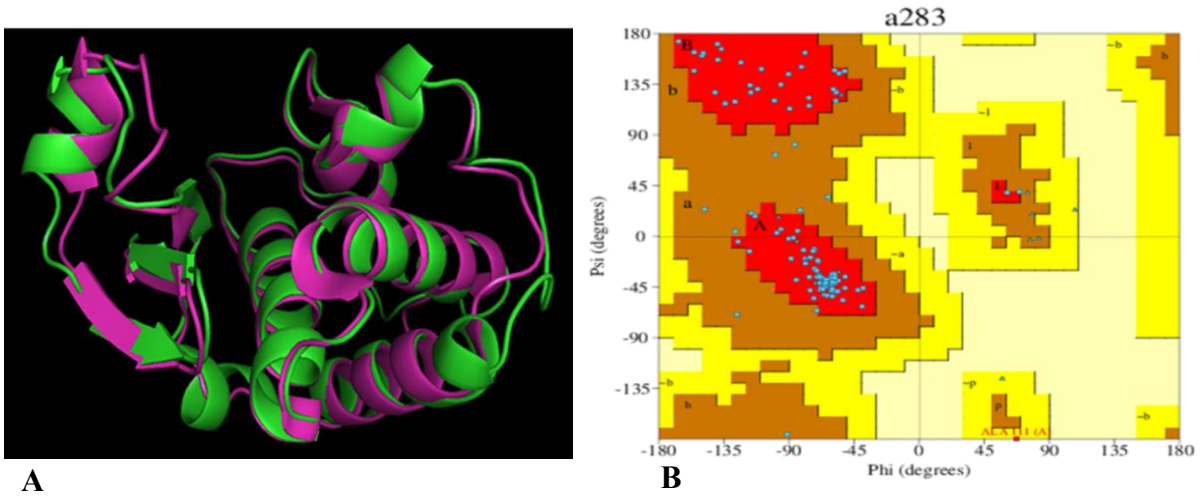


Figure 3.3.5 (A) *Rba. johrii* and *Bacillus* template models superimposed based on Cα position using PyMol where green indicates SleR and magenta indicates SleB (B) Ramachandran plot analysis of SleR of *Rba. johrii.*

3.3.1.4 *In silico* structure comparison, similarities and catalytic residue identification of SleR

Globular and 3D structure of SleR of *Rba. johrii* showing the conserved catalytic glutamate residue exactly in the same location as present in the germination lytic transglycosylase SleB at catalytic domain of *B. anthracis* and the catalytic groove also appears similar (Fig. 3.3.6). Sle protein was superimposed to the Sleb protein crystal structures for comparison. First the template of SleB from *B. cereus* was used here the superimposition showed the catalytic residue glutamate at 128 position of Sle is conserved exactly in the groove between the α and β domains as in SleB of *B. cereus*. This groove is sorrounded by aromatic amino acid residues Phe167, Phe209, Trp218, His231, Phe233 conserved in all SleB (Table 3.3.2) (Fig. 3.3.7) type proteins. When the template of SleB from *B. anthracis* ATCC 14578 was used it was noted that the amino acid residues Tyr153, Gly164 are conserved but replaced by same functional group and Trp218 is conserved (Table 3.3.3; Fig. 3.3.8). Also the catalytic, signature, aromatic residues Arg147, Val148, Glu132, Pro154, Phe167 were conserved in the exact location around the catalytic groove.

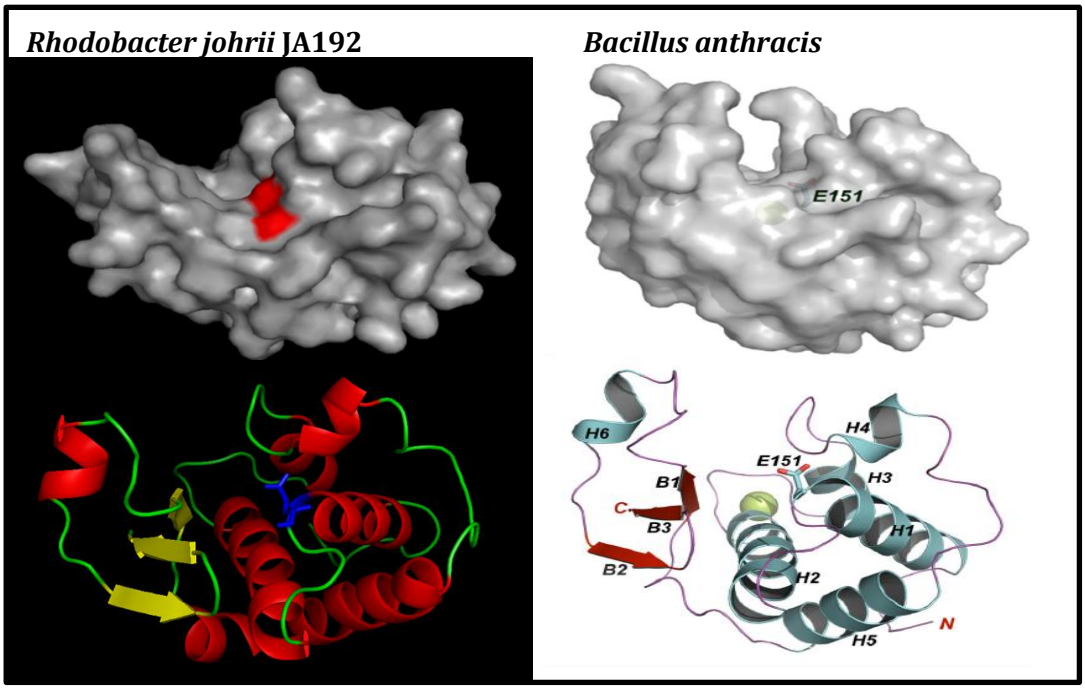


Figure 3.3.6 Globular and 3D structure of SleR of *Rba. johrii* showing the conserved catalytic glutamate residue which is present in catalytic domain of germination-specific lytic tansglycosylase SleB in *B. anthracis*.

Table 3.3.2 Conserved aromatic amino acid residues lined in groove between the α and β domains between *Rba. johrii* and *B. cereus*.

SleB (*B. cereus*) residues	Sle (*Rba. johrii*) residues	Status
Glutamic acid(E) 157	Glutamic acid(E) 128	Conserved catalytic residue
Tyrosine(Y) 232	Histidine(H) 208	Not conserved
Tyrosine(Y) 231	Threonine(T) 207	Not conserved
Phenylalanine(F) 233	Phenylalanine (F) 209	Conserved
Tryptophan(W) 242	Tryptophan(W) 218	Conserved
Phenylalanine(F)196	Phenylalanine(F) 167	Conserved
Histidine(H) 255	Histidine(H) 231	Conserved
Phenylalanine(F) 257	Phenylalanine(F) 233	Conserved

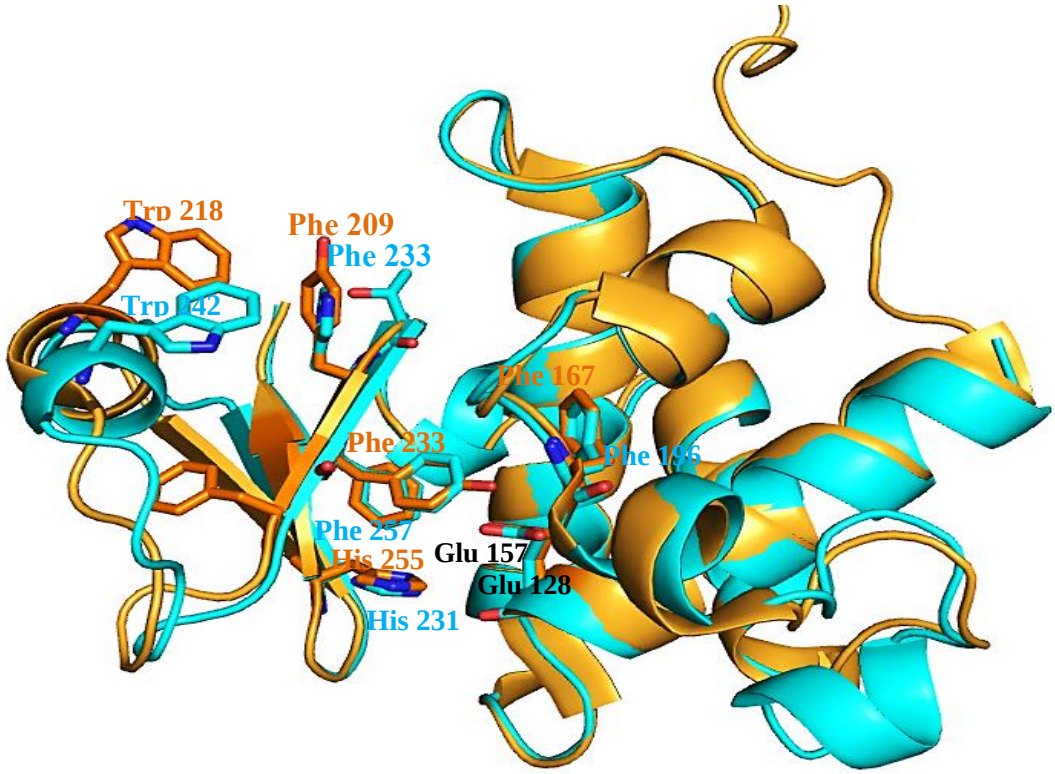


Figure 3.3.7 Superimposition of SleR (orange) of *Rba. johrii* and SleB (cyan) of *B. cereus* backbones showing the side chains of structurally conserved aromatic acid residues (Phe167, Phe209, Trp218, His231, Phe233) present in the catalytic groove along with the catalytic residue (Glu128) and conserved amino acids are written with the same colors.

Table 3.3.3 Conserved amino acids important for binding with muramic-δ-lactam in *Rba. johrii* compare with *B. anthracis*.

SleB(*B. anthracis*) residue	Sle(*Rba. johrii*) residue	Status
Glutamic acid(E) 151	Glutamic acid(E) 128	Conserved
Glutamic acid(E) 155	Glutamic acid(E) 132	Conserved
Tryptophan(W) 236	Tryptophan(W) 218	Conserved
Arginine(R) 170	Arginine(R) 147	Conserved
Valine(V) 171	Valine(V) 148	Conserved
Phenylalanine(F) 176	Tyrosine(Y) 153	Functional replacement
Proline (P) 177	Proline (P) 154	Conserved
Proline (P) 187	Glycine(G) 164	Functional replacement
Phenylalanine(F) 190	Phenylalanine(F) 167	Conserved

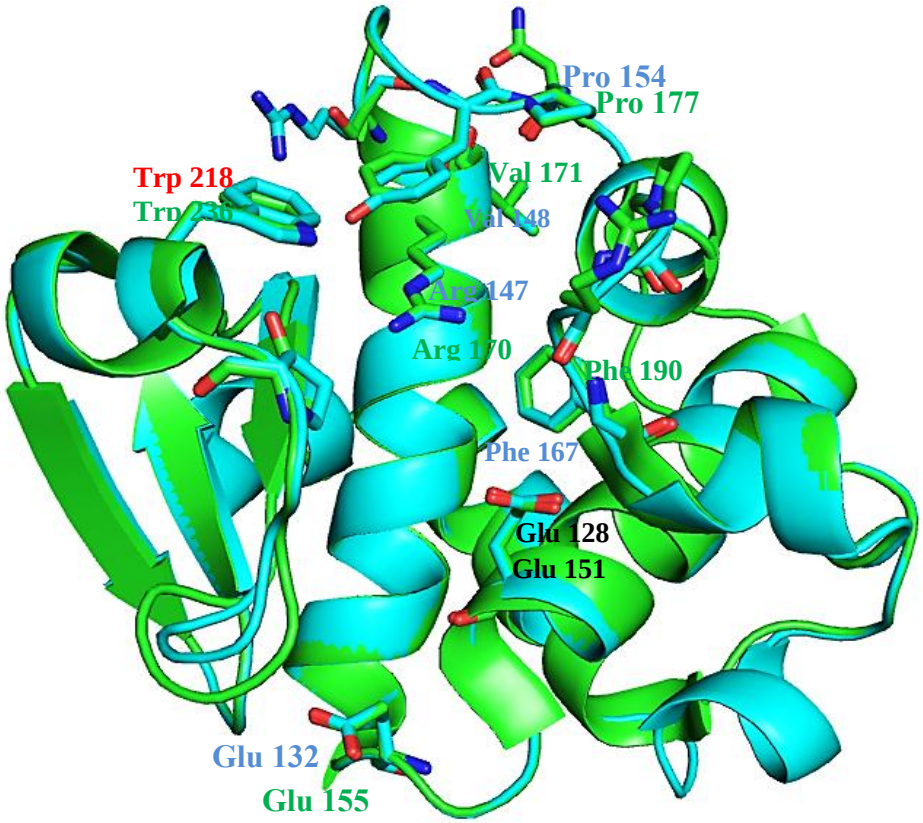


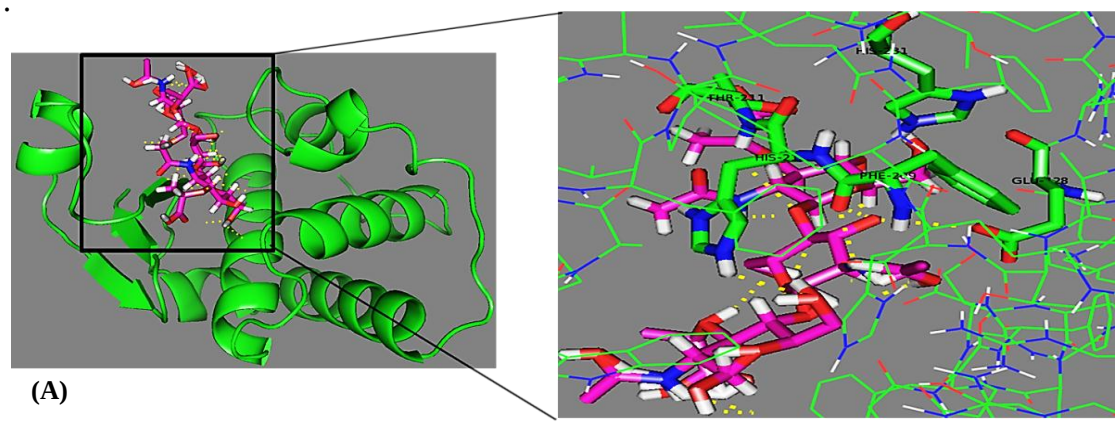
Figure 3.3.8 Superimposition of SleR (cyan) of *Rba. johrii* and SleB (green) of *B. anthracis* backbones showing the side chains of structurally conserved residues (Tyr153, Gly164replaced by same functional group amino acid and Trp218, shown in red colour, these amino acid are responsible for binding to muramic-δ–lactam (MAL)) and residues (Glu132, Arg147, Val148, Pro154, Phe167) are present in exact same location in the groove in both proteins.

3.3.1.5 Molecular docking

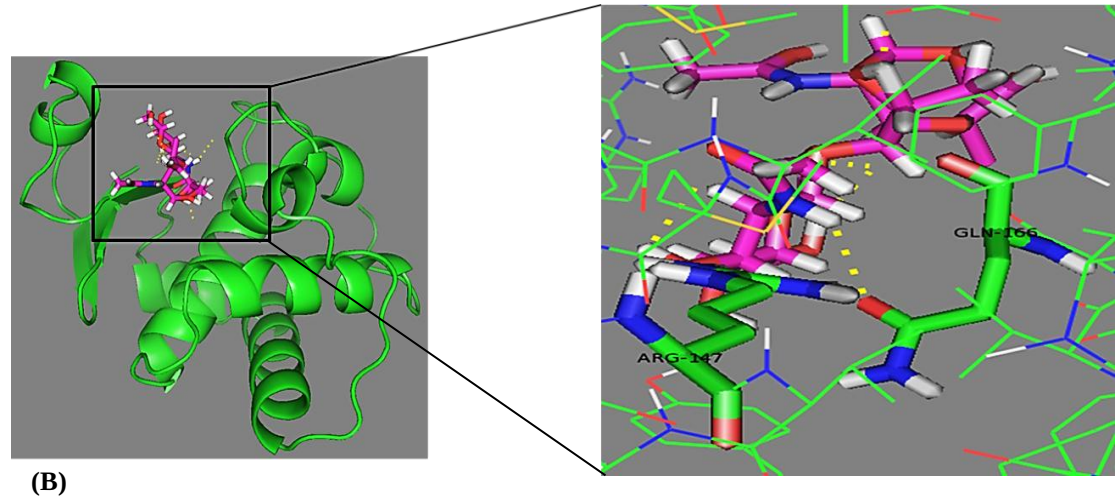
As the Sle protein is predicted to be a spore cortex lytic enzyme therefore its predicted substrate is peptidoglycan which is alternate N-Acetylglucosamine, N-Acetylmuramic acid and Muramic δ-Lactam, polymer. Therefore we have docked with the available peptidoglycans, to find out the binding amino acids and -N-Acetylmuramic acid, NAG-NAM-(Pentapeptide), muramic-δ-Lactam, NAM-NAG-NAM, (1,6-Anhydro-N-acetylmuramic acid) as substrate were used for docking studies. The interaction of these substrates with Sle protein in *Rba. johrii* showed their binding with Phe209, Arg147, Thr211, Gly128, Ser168, Gly166, Gly165, His231, His210 and Glu128 of the protein with their lowest binding energies (Table 3.3.4). All these residues are present in the predicted substrate binding groove and also near to Glu128 (Fig.

3.3.9). The *in silico* characterization of Sle protein of *Rba. johrii* showed properties similar to spore cortex lytic enzyme and since it is different from SleB protein commonly found in members of the phylum Firmicutes, we propose the name Spore Cortex Lytic Enzyme of *Rba. johrii* (SleR) protein of *Rba. johrii.*

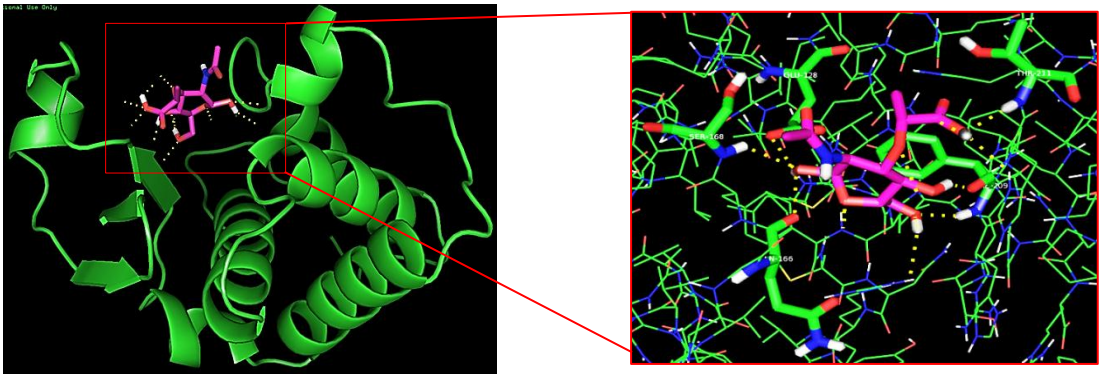
Figure 3.3.9 Molecular docking of (A) trisachharide (NAM-NAG-NAM) (B) 1, 6- Anhydro-N-acetylmuramic acid (C) N- acetylmuramic acid (D) NAG-NAM-(Pentapeptide) (E) Muramic lactam (F) N-Acetylglucosamine with Sle protein of *Rba. johrii*.



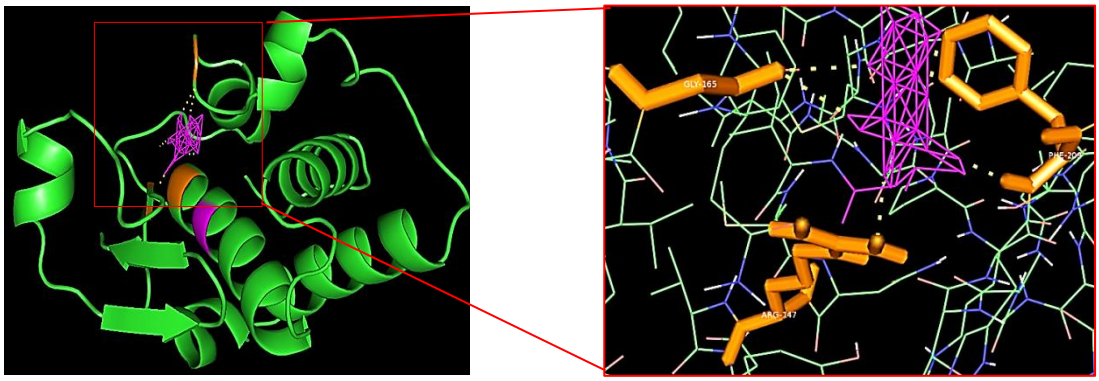
(A)



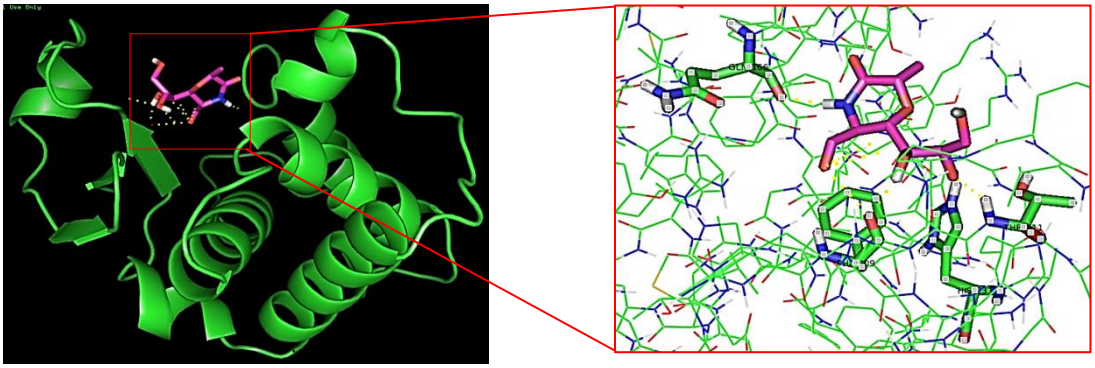
(B)



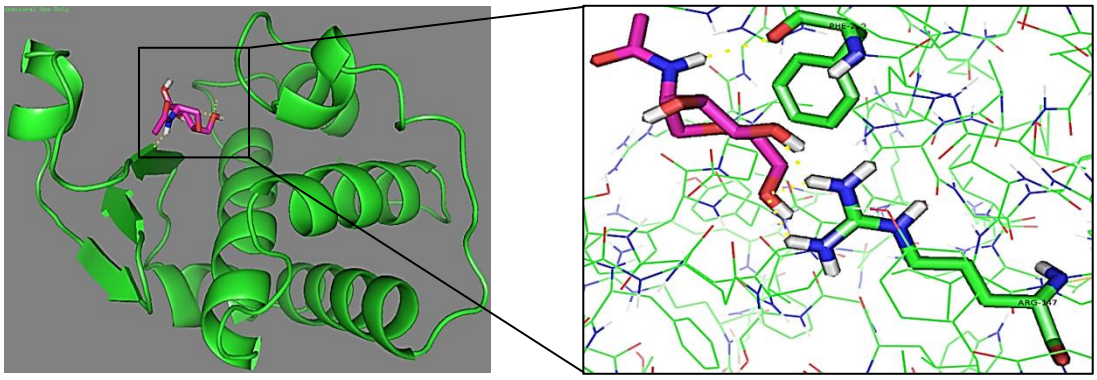
(C)



(D)



(E)



(F)

Table 3.3.4 Interaction (Docking) monomers and polymeric substrates with different amino acid residues of SleR protein.

Substrates	Amino acid residues biding with substrates	Binding energy (Kcal/mol)
N-Acetylglucosamine	Phe-209, Arg-147	-5.1
N-Acetylmuramic acid	Phe-209, Thr-211, Gly-128, Ser-168, Gly-166	-5.5
NAG-NAM-(Pentapeptide)	Phe-209, Arg-147, and Gly-165	-6.4
Muramic lactam	Phe-209, Gly-166, Thr-211, His – 231	-5.3
NAM-NAG-NAM	Thr-211, His -210, His -231, Phe-209, Glu -128	-11.0
(1, 6- Anhydro-N-acetylmuramic acid)	Gly – 166, Arg – 147	-8.1

3.3.2 Biochemical (*In vitro*) characterization of putative SleR protein

3.3.2.1 Molecular cloning of SleR

A truncated 674 bp. *SleR* gene coding for 24-240 amino acid was amplified from *Rba. johrii* genomic DNA by using gene specific primers (Fig. 3.3.10A). Vector pET SUMO Plasmid was digested with BamHI and XhoI restriction enzymes (Fig. 3.3.10B) as well as *SleR* PCR amplified product. The amplicons were cloned into pET SUMO vector. After screening the positive clones through colony PCR, plasmid was isolated from one of the positive clone which was further confirmed through restriction digestion (Fig. 3.3.11). The orientation and sequence of open reading frame was also confirmed through DNA sequencing. The pET-SUMO-$SleR_{24-240}$, construct was transformed into *E. coli* Rosetta (DE3) expression cells. Subsequent solubility analysis has shown that approximately 70% of the recombinant protein was obtained in the soluble fraction

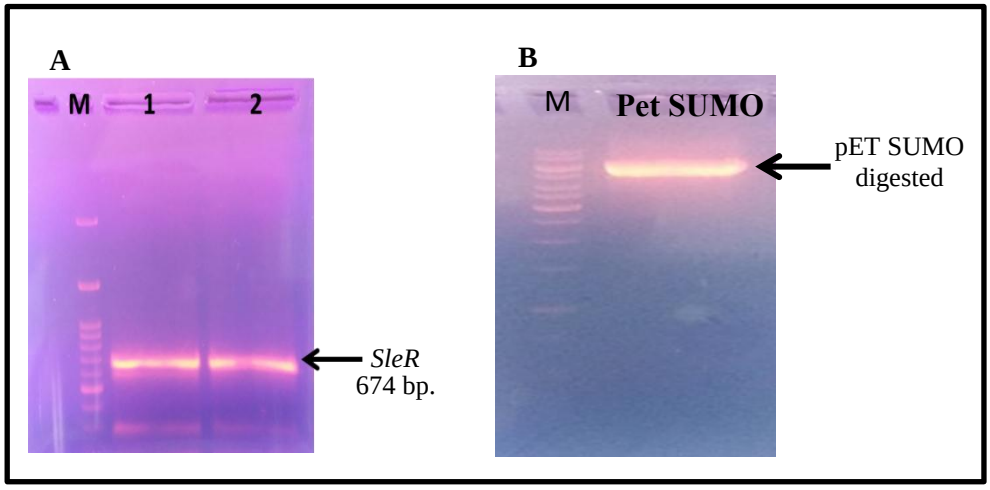


Figure 3.3.10 (A) PCR amplified product of 674 bp. of *SleR* gene without signal peptide (B) Pet SUMO Plasmid digested with BamHI and XhoI restriction enzymes. M-1Kb. Marker.

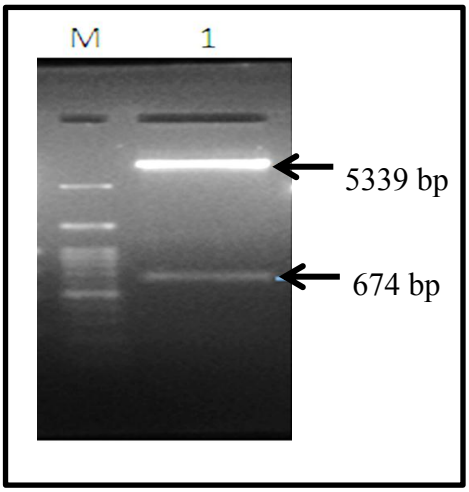


Figure 3.3.11 Confirmation of positive clone by double digestion of *SleR* in pET SUMO

3.3.2.2 Overexpression and purification of recombinant SleR of *Rba. johrii*

First 23 amino acid of N-terminal of $SleR_{24-240}$ protein, which is predicted to be a signal sequence was deleted and 24 to 240 amino acid containing C-terminal hydrolase domain within a construct pET-SUMO-$SleR_{24-240}$ vector was overexpressed as along with histidine and Small Ubiquitin-like modifier tag (His6–SUMO) at N-terminal was fused and overexpressed in *E. coli* Rosetta (DE3) (Fig. 3.3.12).

Overexpressed protein $SleR_{24-240}$ with N-terminal His6-SUMO tag remained in soluble form which was purified with Ni-NTA affinity followed by cation-exchange chromatography was confirmed through western blotting using antibodies against the His-6 tag and its sequence from MALDI-TOF MS- MS matches (Fig. 3.3.12) with the sequence of Sle protein of *Rba. johrii* and also checked in SDS gel (Fig. 3.3.13). The tag was removed by DeSUMOylases cysteine protease treatment and the SDS-PAGE shows the expected molecular mass of SleR (~24 KDa) after digestion of *Rba. johrii* (Fig. 3.3.14). The digested protein was further purified with cation-exchange chromatography resulting in a pure protein free from His6 –SUMO tag.

Query	Start – End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Peptide
38	49 – 59	1215.7808	1214.7735	1214.5819	158	0		K.VSDGSSEIFFK.I
2	63 – 68	743.5571	742.5498	742.4450	141	1		K.TTPLRR.L
19	69 – 76	981.6598	980.6525	980.5113	144	1		R.LMEAFAKR.Q + Oxidation (M)
3	80 – 85	766.4635	765.4562	765.3327	161	0		K.EMDSLR.F + Oxidation (M)
80	80 – 92	1615.0028	1613.9955	1613.7872	129	1		K.EMDSLRFLYDGIR.I
13	86 – 92	883.6070	882.5997	882.4599	158	0	39	R.FLYDGIR.I
21	146 – 155	1040.7497	1039.7424	1039.5774	159	0		R.NALGTLPAQR.L
130	146 – 169	2474.8220	2473.8147	2473.3765	177	1	71	R.NALGTLPAQRLEQVAAAVARPEAK.S

Query	Start – End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Peptide
65	156 – 169	1453.0488	1452.0415	1451.8096	160	0		R.LEQVAAAVARPEAK.S
30	184 – 195	1081.7577	1080.7504	1080.5676	169	1		R.GAKAADGAPAPR.Y
6	187 – 195	825.5500	824.5427	824.4140	156	0		K.AADGAPAPR.Y
77	196 – 209	1593.0215	1592.0143	1591.7518	165	0		R.YDEDWLASQPVAAK.D
127	196 – 215	2353.4363	2352.4291	2352.0546	159	1		R.YDEDWLASQPVAAKDSDEWK.C
51	216 – 226	1314.8728	1313.8656	1313.6438	169	0		K.CLATALYFEAR.G
93	227 – 243	1849.2403	1848.2330	1847.9200	169	0		R.GESIQGQFAVAEVIMNR.V
94	227 – 243	1865.2500	1864.2427	1863.9149	176	0	121	R.GESIQGQFAVAEVIMNR.V + Oxidation (M)
148	227 – 258	3478.2486	3477.2414	3476.7293	147	1		R.GESIQGQFAVAEVIMNRVDRPGYPGSICGVVR.Q + Oxidation (M)
82	244 – 258	1632.1079	1631.1007	1630.8250	169	0	61	R.VDRPGYPGSICGVVR.Q
111	259 – 275	1961.2825	1960.2752	1959.9149	184	0	76	R.QGGQFSFMFDGKPETIR.E + Oxidation (M)
124	259 – 277	2202.4270	2201.4198	2201.0575	165	1		R.QGGQFSFMFDGKPETIREK.A
125	259 – 277	2218.4400	2217.4328	2217.0525	172	1		R.QGGQFSFMFDGKPETIREK.A + Oxidation (M)
31	286 – 296	1083.8156	1082.8083	1082.6270	167	0		K.IAALMLAGAPR.Q
33	286 – 296	1099.8082	1098.8009	1098.6219	163	0		K.IAALMLAGAPR.Q + Oxidation (M)
58	297 – 308	1396.9498	1395.9426	1395.7008	173	0		R.QLTQGATHFHTR.A
24	309 – 317	1049.7594	1048.7522	1048.5679	176	0	43	R.AVRPGWAHR.F
64	309 – 320	1450.0494	1449.0421	1448.7902	174	1		R.AVRPGWAHRFPR.T
39	321 – 331	1219.8679	1218.8606	1218.6509	172	0		R.TAAIGAHLFYR.Q
83	321 – 336	1646.1420	1645.1347	1644.8372	181	1		R.TAAIGAHLFYRQPGGS.-

>WP_069331589.1 cell wall hydrolase [Rhodobacter johrii) (without signal peptide)

DVTVSQSNDPSVNLGGQLSALLGAERNALGTLPAQRLEQVAAAVARPEAKSEAPVKAVAKRGSRGAKAAD
GAPAPRYDEDWLASQPVAAKDSDEWKCLATALYFEARGESIQGQFAVAEVIMNRVDRPGYPGSICGVVRQ
GGQFSFMFDGKPETIREKAAFQRAGKIAALMLAGAPRQLTQGATHFHTRAVRPGWAHRFPRTAAIGAHLF
YRQPGGS

Figure 3.3.12 MALDI TOF MS-MS analysis of purified protein of His_6-SUMO-$SleR_{24-240}$. Matched peptides are shown in bold red.

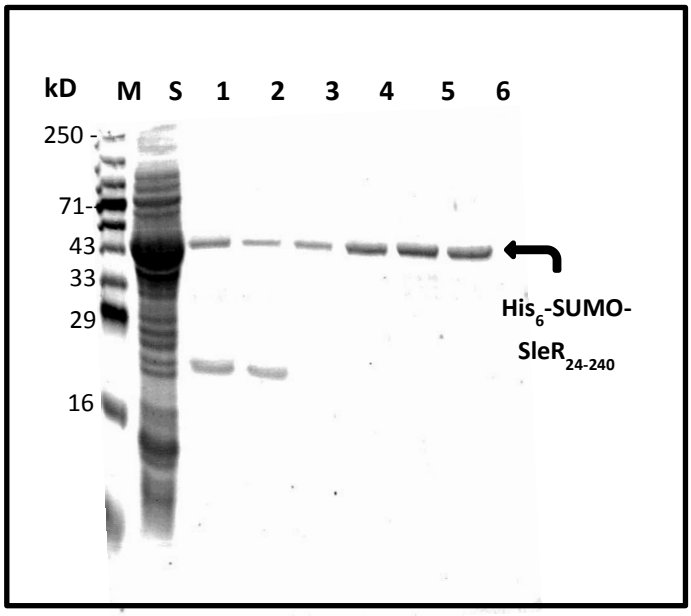


Figure 3.3.13 SDS-PAGE of Soluble SleR here overexpressed protein His_6-SUMO- $SleR_{24-240}$ showed by black arrow, M- protein marker ; kDa, S- supernatant, Lanes - 1-6 - Ni affinity eluate increased at each lane 50 mM from 100 mM to 350 mM imidazole.

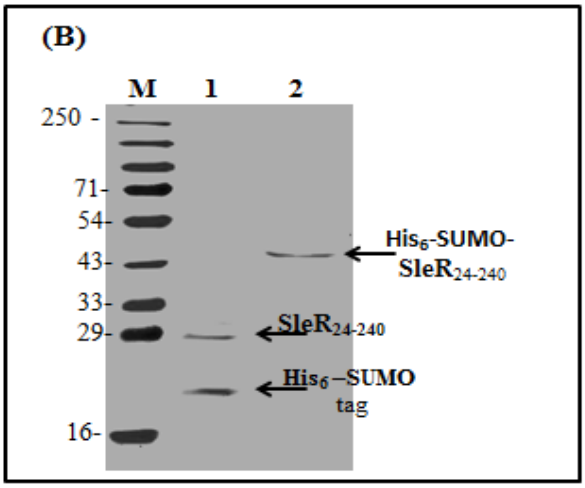


Figure 3.3.14 M- marker 1- DeSUMOylases cysteine protease digestion 2 - Ni - affinity eluate.

3.3.2.3 Substrate specificity and enzyme activity of spore cortex lytic enzyme (SleR)

Enzyme assay was done from purified $SleR_{24-240}$ protein with the spore sacculi isolated from *B. subtilis* FW2 was incubated with recombinant $SleR_{24-240}$ protein for 45 min here loss of 50% of substrate was observed at OD_{600} (Fig. 3.3.15). This shows that purified SleR cleaves *B.*

subtilis spore cortex. On the other hand OD loss was not observed when the vegetative cell wall (peptidoglycan) from *B. subtilis* was used as substrate for $SleR_{24-240}$ protein (Fig. 3.3.16).

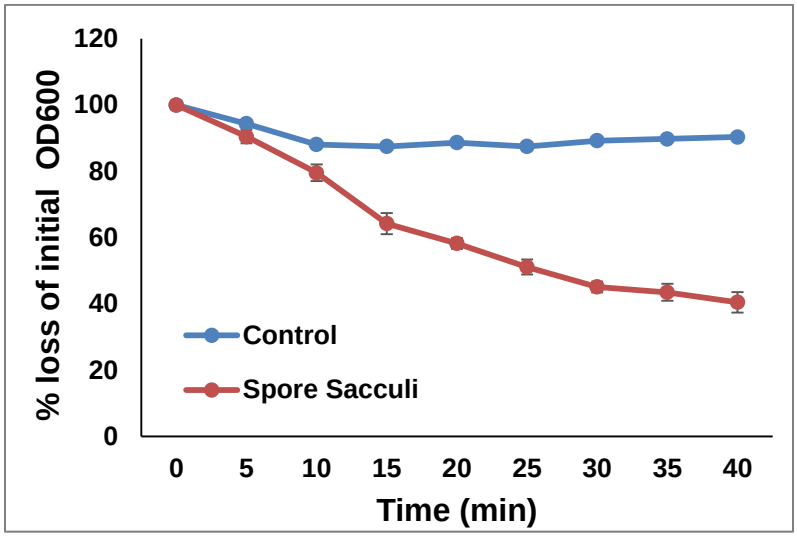


Figure 3.3.15 Enzyme assay, cleavage of spore sacculli isolated from *B. subtilis* using $SleR_{24-240}$ protein with spore sacculi is indicated in red line and blue line indicates control reaction in which substrate was untreated and loss of OD was not observed.

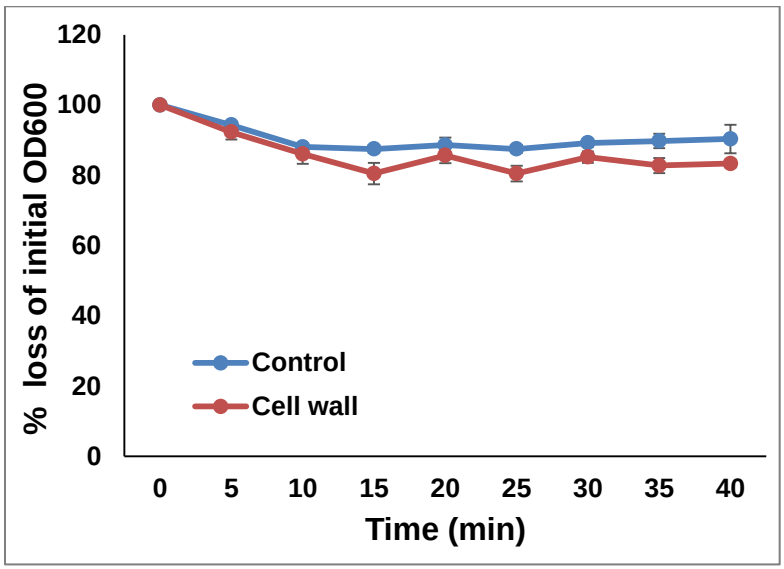


Figure 3.3.16 Enzyme assay done taking cell wall peptidoglycan with red line and control reaction with blue line here no protein was added.

3.4 *In vivo* expression profile of SleR protein in different growth phases and cell morphology & differentiation of *Rba. johrii*

To study the expression levels of SleR during growth of *Rba. johrii*, growth curve was determined (Fig. 3.4.1). RNA from the cells of different growth phases (lag, mid-log, stationary and decline) was isolated. The differential expression of SleR was studied by qRT-PCR at different growth phases of *Rba. johrii*. *Sler* gene was normalized to the 16S rRNA housekeeping gene and lag phase *Sler* expression was taken as a control. There was no significant difference in the expression of *Sler* till the stationary phase as compared to lag phase (Fig. 3.4.2). However, during late stationary phase the expression of *Sler* was increased drastically (~4.5-fold) and eventually reduced during late decline phase. Further, *Sler* expression levels under heat stress conditions at late stationary and late decline phases were also checked the compared with expression levels at late stationary and late decline phases under normal growth conditions (without heat stress). Only a marginal difference in the expression levels of *Sler* was observed under both the heat stress and normal growth condition (~0.5-fold). These results confirm that *Sler* is expressed when the culture produces endospore-like dormant structures during late stationary phase of *Rba. johrii* growth and on the other hand, rule out the possibility of its heat induced expression.

We examined the *Rba. johrii* culture development and differentiation during the different growth phases parallelly to correlate with *Sler* expression analysis. Cells were examined through the microscopy, Schaeffer and Fulton staining, at early growth phases lag to stationary phase any differentiation was not noted morphologically. As the expression of *Sler* started spiking in late stationary phase the cells were started differentiating into spore (Fig. 3.4.2). At late decline phase *Sler* expression decreased, here the cells has mature spore like refractile bodies.

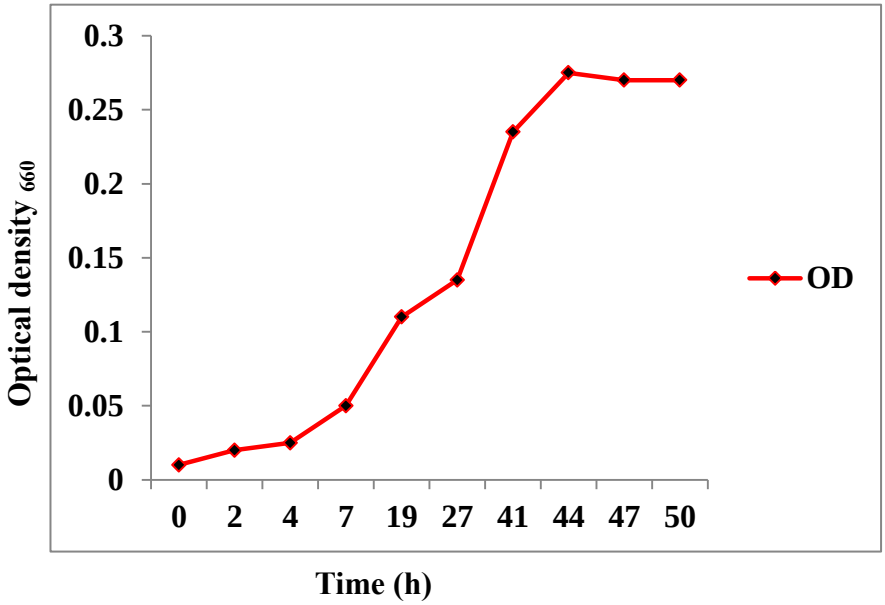


Figure 3.4.1 Growth curve of *Rba. johrii*

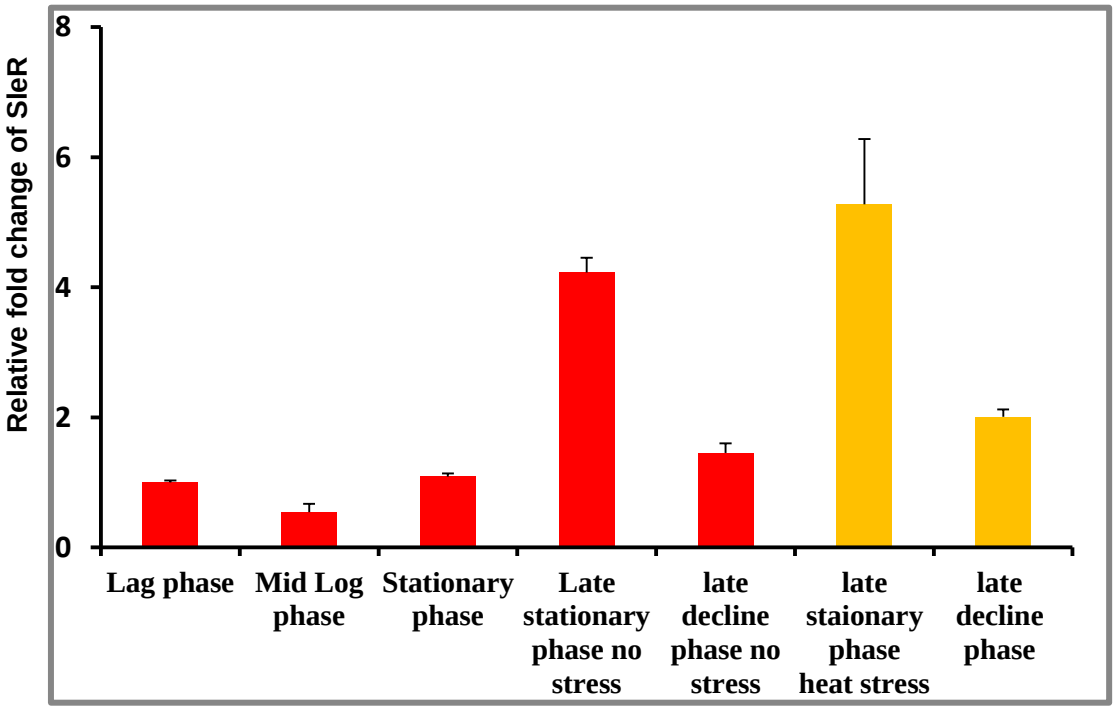


Figure 3.4.2 qRT-PCR results show the differential expression of SleR at different growth phases of *Rba. johrii*. Bars represent mean standard deviation of three independent experiments. Red colour indicates absence of any stress and orange colour indicates presence of heat stress.

DISCUSSION

4. Discussion

Favorable environmental conditions lead to the completion of bacterial vegetative cycle normally through the binary fission (Wang and Levin, 2009). Natural habitats are highly dynamic that face frequent fluctuations to physio-chemical and environmental conditions during the period of time (Woodward *et al.*, 2016; Brooks *et al*., 2011). Microorganisms developed the responses due to adaptations under these unfavorable conditions using many survival strategies which either manifest as a modification of their lifestyle or transient escape routes (Brooks *et al*., 2011; Jones *et al*., 2010; Dworkin and Shah 2010; Spector and Kenyon 2012; Lebre *et al*., 2017). These include many mechanisms like viable but nonculturable (VBNC) state where cells become metabolically inactive in adverse conditions (Nowakowska *et al*., 2013; Xu *et al*., 1982). Cysts which are thick walled structures containing capsular slime reported in *Azotobacter* (Socolofsky and Wyss, 1962) and in *Serratia ureilytica* (Filippidou *et al*., 2019). Exospores are structures formed outside the cell in bacteria like *Methylosinus* spp. (Reed *et al*., 1980; Reed and Dugan 1979) also common among many filamentous *Streptomyces* and a few other members of *Actinobacteria* (Ryding *et al*., 1998). Endospores are tough dormant bodies found among the members of the phylum Firmicute, wherein *Bacillus subtilis* has been the model organism (Hutchinson *et al*. 2016; Piggot and Hilbert 2004). Dormancies like exospore, endospore and cyst are well known and studied extensively (Piggot and Hilbert 2004; Bobek *et al.*, 2017).

There are reports for the existence of endospores among non-Firmicute members like in *M. xanthus* (Kaiser 2006; Kroos 2007) *C. burnetii* (McCaul and Williams, 1981), *S. marcescens* (Ajithkumar *et al*., 2003) few *Mycobacterium* spp. (Singh *et al*., 2010; Ghosh *et al*., 2009; Lamont *et al*., 2012), *Rhodopseudomonas palustris* (Venkidusamy and Megharaj, 2016). *Rba. johrii* produces endospore like refractive structures with some similarity and differences to the

Firmicute endospores (Girija *et al.*, 2010). However, these cases have always been met with skepticism as they challenge the well-accepted finding that endospores are confined to some Firmicutes members. For example, there have been consecutive studies which have found endospores in non-Firmicute *Mycobacterium* spp. (Singh *et al.*, 2010; Ghosh *et al.*, 2009) followed by failures to reproduce these results (Traag *et al.*, 2010) and again the demonstration of their presence (Lamont *et al*., 2012). Recently, a study was unable to confirm the refractive bodies as endospores in *Rba. johrii* and *S. marcescens* based on microscopic and bioinformatic analysis and concluded the refractile bodies to be triacylglycerol granules and cellular debris of respective species (Beskrovnaya *et al.*, 2020).

Dormancy reported among the non-Firmicute members are not studied in depth along with aspects of molecular and genetic machinery therefore limiting our ability to perform the comparative study with that of canonical sporulation for further study of the non-Firmicute members. *Rba. johrii* was selected as a model organism in present study for the insights of endospores and molecular machinery of a non-firmicute sporogenesis. In this study, the endospore like refractive bodies are physiologically and structurally characterized. Comparative genome analysis helped in the identification of gene homologs related to sporulation and signature genes, the important genes identified was characterized and compared to understand its significance in possible sporulation in *Rba. johrii.*

Leads from morphological study

Rba. johrii, a photosynthetic proteobacterium that was isolated from a pasteurized soil produces endospore like structures having morphological similarities with the endospores including refractile structures and stained with malachite green (Girija *et al*., 2010). Sometimes, it is noted that certain characters are lost in bacteria with time therefore, it was necessary to

reconfirm the endospore like refractive bodies of *Rba. johrii.* The refractive bodies of *Rba. johrii* showed characters of endospores like highly refractile bodies and stained with malachite green (Knaysi, 1948; Schaeffer and Fulton, 1933). DAPI binds to the DNA therefore dual stain was done with DAPI and FM 4-64 dye where the refractile bodies were stained with DAPI indicating that refractive body contains DNA and confirms that refractile body are not the artifact (Fig. 3.4), nor they are simple triacylglycerol granules as speculated from cryo-microscopic studies (Beskrovnaya *et al.*, 2020).

PHA granules are accumulated as food storage which appears refractile under phase contrast microscope therefore could be mistaken with the endospores (Chanasit *et al.*, 2016). Therefore, to confirm that the refractive bodies of *Rba. johrii* are not mistaken with the PHA granules, we first stained the cells with Nile red which is lipophilic dye which binds specifically to the lipid inclusions like PHA granules (Spiekermann *et al*., 1999). *Rba. johrii* cells pursued the red fluorescence while the refractile cells don't possess (Fig. 3.5) clearly differentiates PHA granules with the spores. Further, the PHA granules were recovered and dissolve when treated with chloroform (Koller, 2020). *Rba. johrii* chloroform treated cells were stained with malachite green (Fig. 3.6), which further evidence that the refractive bodies are spores. Finally, we have used chemicals like acrylic acid and pentenoic acid to inhibit the PHA granule biosynthesis (Lee *et al*., 2001; Qi *et al.*, 1998) and when supplemented to the mid log phase culture the PHA granules were found to be inhibited completely as observed in Nile red staining and spore like bodies were visible (Fig. 3.7A,B). The staining studies clearly indicates that *Rba. johrii* cells produces endospores like refractive bodies which are well distinguished from PHA granules. We have experimented with one more newly isolated species of the genus *Rhodobacter*; *Rhodobacter alkalitolerans* (Gandham *et al.*, 2018) where such refractive bodies were observed

and were stained with malachite green (Fig 3.13). This result probably explains the wide spread occurrence of such spore like refractive bodies among other anoxygenic phototrophic bacteria not only belonging to the genus *Rhodobacter* but also with the member of the genus *Rhodopseudomonas* (Venkidusamy and Megharaj, 2016).

The fine structure of spore like refractive body was not clear in the previous study (Girija *et al*., 2010) which prompted us to look into an in-depth structure through transmission electron microscope (TEM). The ultra-thin sectioning of spore like refractive body reveals similarities as well as differences with that of *Bacillus* endospore. *Rba. johrii* spore like refractive body possess central core, cortex, additional inner membrane and an outer coat. The inner coat is absent in these bodies, there body outer part has protrude structures like prosthecae (Fig. 3.8.2) which are not seen in *Bacillus*. Spore culture when treated with the Triton-X, the structure was seen to be degraded due to denaturation (Fig. 3.8.2C) of spore coat and other proteinaceous structures, these results confirms that the coat of the spore like refractive body is made up of proteins.

As the current fluorescent staining procedures have certain disadvantages (Table 4.1), we have therefore used a rapid, easy method and chemical dye for staining spore like refractive bodies. The fluorescent spore specific dye has advantage in distinguishing the cryptic spores, as these were misidentified with PHA granules.

Table 4.1: Spore dyes and there drawbacks (Ali *et al.*, 2020)

Reference	Fluorescent Dyes	Drawbacks
Karava *et al*., 2019	Flow cytometric and fluorescence activated cell sorting with nucleic acid fluorescent staining SYBR1,SYBR2 (Invitrogen^{TM})	Two different fluorescent dyes used which binds to nucleic acids
D'Incecco *et al*., 2018	Hoechst 34580	Binds to spores as well as vegetative cells
Probst *et al*., 2012	Propidium monoazide [PMA]	Stains DNA when spore is damaged or permeable (inactivated)
Xia *et al*., 2011	Thioflavin T(Th T)	Dye targets endogenous markers that is amyloid encapsulated in the spores; also stain vegetative cells
Schichnes *et al*., 2006	Acridine orange[AO]	Fluorescent stain for DNA
Tarnowski *et al*.,1991	4′-6′- diamino-2-phenylindole[DAPI]	Fluorescent stain for DNA
Bartholomew *et al*., 1965	Auramine O	Binds to spores, vegetative cells, lipid granules; requires counter staining

While screening some of the zwitterionic diaminodicyanoquinodimethane (DADQ) derivatives for spore staining a chemically synthesized molecule 7,7-(*n*-hexylamino)-8,8-dicyanoquinodimethane (Fig. 3.10) was found to have specificity for the spores of bacteria (Senthilnathan *et al*., 2020) which was termed as "Sporotan".

In our study, it was demonstrated for the first time, the significant use of this DADQ derivative to clearly specifically stains the spore like refractive bodies of *Rba. johrii*. The refractive bodies of *Rba. johrii* were positively stained, neither the vegetative cells nor the PHA

granules were stained with "sporotan" dye (Fig. 3.11A,B). As well as the old culture of *B. subtilis* having spores was stained with sporotan (Fig. 3.11C). The staining procedure was demonstrated to be simple, easy, selective, efficient and cost- effective. The application of stain and the method is to discover the endospores produced among the many bacteria of different phyla, which otherwise are mistaken as PHA granules and largely ignored by microbiologists.

Leads from genomic analysis

Many phenomena thought to be absent in microorganisms have been discovered or rediscovered relatively recently, demonstrating that the scope for such research remains vastly open for thorough investigation (Emerson *et al*., 2008). Bacterial whole genome sequencing and genome mining (Ziemert *et al*., 2016; Alanjary and Medema, 2018; Belknap *et al*., 2020) has headed to the discovery of many novel genes also the clusters of pathways which could then be experimentally demonstrated (Cao *et al*., 2017; Forde and O'Toole, 2013; Kan *et al*., 2018). *Rba. johrii* genome sequencing and analysis was done to find out the genetic content for sporulation.

Genomic characterization of *Rba. johrii* revealed 1075 hypothetical protein encoding genes were predicted to be present in genome of which are not known for their functions (Table 3.2.2). The proteins involving in the synthesis, metabolism and recognition of complex carbohydrates were detected around 510 genes associated with the carbohydrate metabolism is the highest number of genes among the *Rhodobacter* members. Cazyme gene clusters CGC34 and CGC35 are only present in *Rba. johrii* compare to other *Rhodobacter.* Specialty genes found in *Rba. johrii* contain 74 human homolog proteins, antibiotic resistance genes, transporter and drug targets. Twenty-one unique protein families and 11 CRISPR locus were identified in *Rba. johrii*. These set of genes indicates *Rba. johrii* to be a highly metabolically versatile bacterium.

The members of the phylum Planctomycetes were earlier thought to have a proteinaceous cell wall without peptidoglycan (PG) (Fuerst and Sagulenko, 2011; Jogler *et al.,* 2012). However, recent study based on bioinformatics BLAST analysis demonstrated the genome encodes the proteins essential for PG synthesis. The homologues protein were identified at the threshold of identity $\geq$ 20% and coverage $\geq$ 40%. All the peptidoglycan biosynthesis proteins found has the lowest 23% and highest 49% identity. The biochemical analysis and electron microscopic study further definite the occurrence of PG among the members of the phylum Planctomycetes (Jeske *et al*., 2015). Likewise, in this study, based on identification of homologous proteins involve in sporulation of *Rba. johrii*. spore protein homologs were searched according to stage-wise for major proteins. Genome mining of *Rba. johrii* demonstrated the presence and absence of proteins related to sporulation with that of *B. subtilis*. We detected around one hundred proteins with sporulation properties. Out of which, the signature proteins for endosporulation are sigE, sigH, sig F, sig K, spo0A, spoIIAB, spoIIE, SpoIVB, sigG, spoIID, spoIIM, spoIVA, spoVB, spoVG, yqfc, ytvI, sleB, SpoIVB, spoVAD, spoIIG and dapG were uncovered with identities matched to *B. subtilis* sporulation protein ranging from 19-68%. These identified homologous proteins are likely to be involve in endospore like bodies formation of *Rba. johrii*. The similarity and coverage of proteins matched were not considerably high between these two bacteria. These are distantly related therefore at the time of evolution, the exact sequence similarities among these proteins are low while may conserve their major functions. Therefore, the sporulation pathway for *Rba. johrii* may be different but sharing some common stages and proteins with that of *B. subtilis*.

The presence of dipicolinic acid (DPA) and small acid soluble DNA-binding proteins (SASPs) in *Bacillus* spores provides resistance and longevity to the spores (Setlow, 2007). DPA

was absent in the *Streptomyces* spores (Janssen *et al.,* 1958) but some of the thermophilic *Actinomycetes* have DPA (Kalakoutskii *et al.,* 1969) similar to *Bacillus* spores (Huang *et al.,* 2007). DPA biosynthetic pathway and SASP homolog proteins are absent in *Streptomyces* spores (Ensign, 1978) in place of these, other nucleoid associated proteins incorporate into nucleoid structure and protect the spores (Chater, 2011). Likewise, in this study small acid soluble proteins (SASPs) and DPA synthesis pathway protein encoding genes are absent in *Rba. johrii* genome. The absence of DPA and SASP encoding genes in *Rba. johrii* indicates that there might be some other novel proteins with similar function like that observed in *Streptomyces* spores having alternate proteins with similar function.

Spo0A is a major regulator of sporulation which initiates the sporulation through a rely of signaling cascade proteins in *Bacillus* (Burbulys *et al*., 1991). All these protein encoding genes were found to be conserved and Spo0B was absent in *Rba. johrii* genome. Spo0B protein could have been replaced by some other protein with similar function. On the basis of protein identified in starvation state-the putative signaling pathway for spore initiation in *Rba. johrii* was predicted (Fig. 4.1).

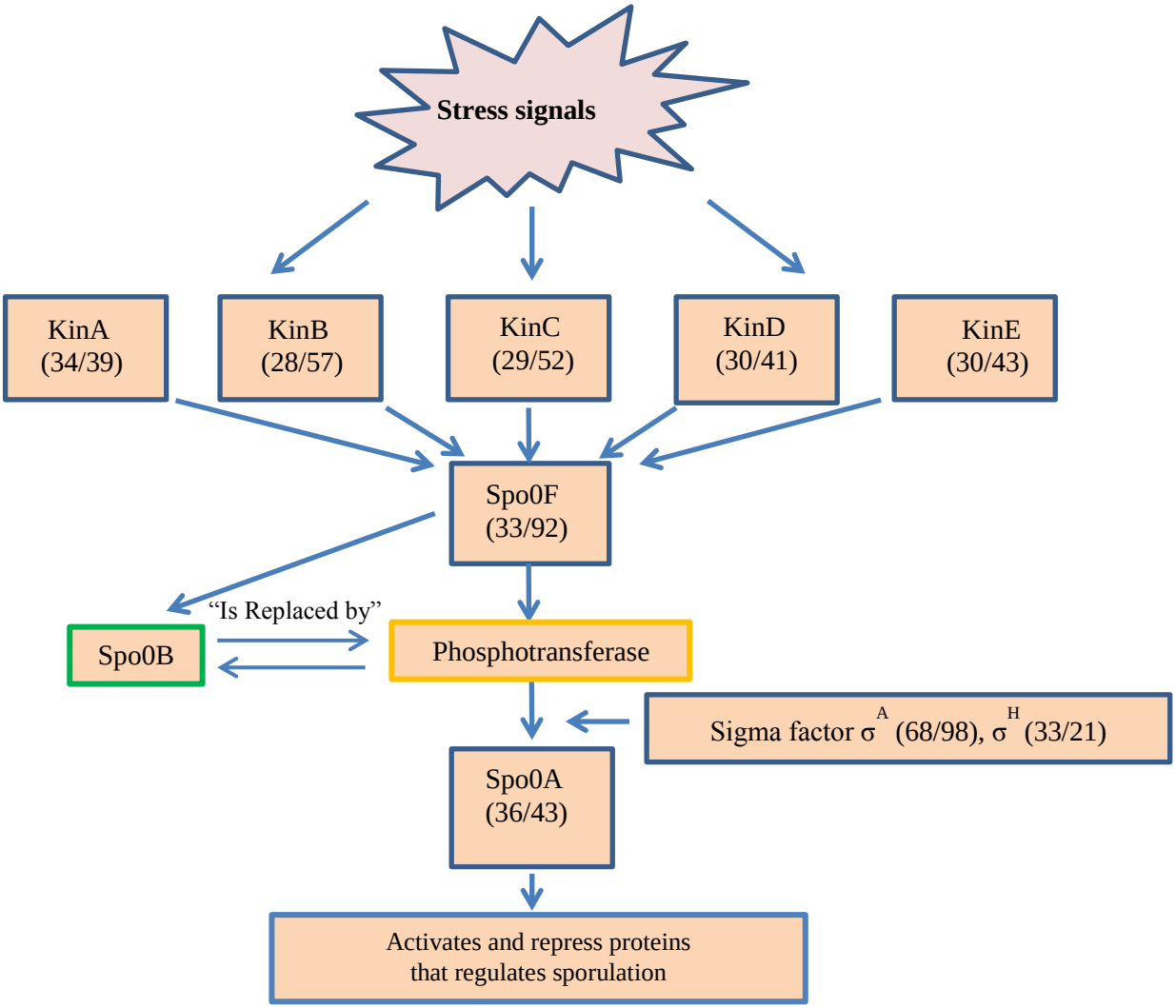


Figure 4.1 Flow chart of putative pathway of spore initiation signaling cascade in *Rba. johrii* on the basis of identified homolog proteins of *Rba. johrii*. Green box shows the absence of Spo0B protein; Orange box for alternate phosphotransferase in *Rba. johrii*. Parenthesis values of amino acid given in identity/protein coverage in *Rba. johrii* when compared to *Bacillus subtilis* subsp. *subtilis* 168.

In other sporulation stages, there were some gene coding proteins which are present and some are absent which are given in result part but on the whole, major proteins involved in sporulation of *B. subtilis* were also found in *Rba. johrii*. Over five hundreds of genes are responsible for endospore formation in *B. subtilis* (Shi *et al.*, 2020) out of which in a study around forty one sporulation gene homologs have been found in the *Rba. johrii* genome in that many involved in other roles like signalling, cell division and development (Beskrovnaya *et al.*,

2020). There other genes were unable to cover due to the cut off kept was identity 30% and coverage 60%, but in our study we were able to identify around hundreds of proteins (Table. 3.2.13) mostly are sporulation proteins and do not have functions in normal cell-functioning, along with low identity coverage ranging from 19% to 68%, although the identity is low but as in case of planctomycetes where the peptidoglycan biosynthesis proteins were found in genome having the low 23% - 49% identity were accepted also the biochemical analysis and electron microscopic study further definite the occurrence of PG among the members of the phylum Planctomycetes (Jeske *et al*., 2015). This could be low in our case because *Rba. johrii* is distantly related to *B. subtilis*, therefore their exact sequence similarities are low with conservation of major functions among them.

Interestingly, out of thirty-six major coat proteins including outer and inner coat proteins of *B. subtilis*, only fourteen were matched in *Rba. johrii*. This result suggests that the endospore-like refractive structure of *Rba. johrii* might be having a different spore protein and coat composition, the triacylglycerides are the probable components of these (Beskrovnaya *et al.*, 2020). The ultra-thin sections of endospore-like refractive structures of *Rba. johrii* (Fig. 3.8) indicate absence of a multi-layered coat and an inner coat. Only one outer coat which is thick is present and the same is throw back in the genome mining here the homologs of most coat proteins are absent.

Leads from characterization of identified putative spore cortex lytic enzyme (SleR) protein (*In vitro* and *In vivo)*

As experimental analysis would help to understand whether these identified one hundred putative proteins also have similar properties to that of *B. subtilis* spore proteins. The identified SleR protein from *Rba. johrii* is a homolog of *B. subtilis* spore cortex lytic enzyme (SleB) was

characterized. The SleB protein of *B. subtilis* is well-characterized and validated protein of the endospore (Boland *et al*., 2000; Li *et al.,* 2012). This protein in *Bacillus* is expressed during sporulation as an inactive form and localized on the cell outer cortex of spore. During germination the enzyme is activated and hydrolyses the peptidoglycan of the spore cortex (Moriyama *et al.,* 1999).

When the multiple sequence alignment of SleR was done with that of *Bacillus* spp. SleB protein the N-terminus has variation but the C-terminus having catalytic domain was highly conserved (Fig. 3.3.3). SleR protein has conserved hydrolase 2-super family (pfam07486) catalytic domain mostly found in SleB proteins of many *Bacillus* spp. (Jing *et al*., 2012). Another domain which is Peptidoglycan (PG)-binding domain in N-terminal region was not conserved in SleR of *Rba. johrii.* The SleB protein of *B. subtilis* without the N- terminal region having the peptidoglycan (PG) binding domain was found to be functional therefore its absence does not have an impact on the function of the protein (Heffron *et al*., 2011) therefore the N-terminal region does not have significant role for the protein function. Spore cortex lytic enzyme CwlJ in *Bacillus* spp., lacks the N-terminal peptidoglycan (PG)-binding domain and possess only C-terminal hydrolase sphere (Ishikawa *et al*., 1998). Similarly, in the SleR protein of *Rba. johrii* the absence of peptidoglycan domain is found but C-terminal hydrolase domain is present therefore this protein may function like a SleB protein.

The SleR protein hydrolase catalytic domain multiple sequence alignment with other SleB *Bacillus* spp. has more conserved amino acid including the glutamate catalytic residue (Fig. 3.3.3). We have identified SleB specific EXRGE sequence motif which is having the two alternating glutamate residues (at 128 and 132 position) a signature sequence in SleB type proteins (Jing *et al*., 2012). Secondary structure of SleR of *Rba. johrii* has the conserved

glutamate catalytic residue present in substrate binding groove similarly as in the germination – specific lytic transglycosylase (SleB) catalytic domain in *B. anthracis* (Fig. 3.3.6)

Superimposition of SleR from *Rba. johrii* and SleB from *B. cereus* showed that aromatic amino acid residues lined between the α and β domain groove (Fig. 3.3.7) is responsible for binding and cleavage of spore cortex peptidoglycan (Li *et al.,* 2012). When superimposition of SleR from *Rba. johrii* and SleB from *B. anthracis* showed the three amino acids which are important for binding with muramic-δ-lactam (Jing *et al.,* 2012) are also observed in *Rba. johrii* (Fig. 3.3.8). As well as showed the conserved amino acids are present around the catalytic binding groove and even the glutamate residue is located at exact position as reported in other SleB proteins (Li *et al*., 2010; Jing *et al*., 2012). The predicted substrates (peptidoglycan monomer and polymer) were docked with the identified SleR protein. Docking studies showed that as earlier reported in case of SleB protein where the amino acids present in the groove and the glutamate residue binds with the peptidoglycan (Li *et al*., 2010; Jing *et al*., 2012) similarly those conserved amino acid residues of SleR protein has also bind to the same peptidoglycan residues (Fig. 3.3.9). Overall *in silico* characterization confirms that identified putative SleR protein from *Rba. johrii* has the properties of a spore cortex lytic enzyme and therefore this protein named as spore cortex lytic enzyme from *Rba. johrii* as SleR.

The hydrolase enzyme assay for SleR protein was performed using spore cortex sacculi extracted from *B. subtilis* FW2 as substrate and loss in the OD indicates the cleavage of peptidoglycan at least from *B. subtilis* spore cortex (Fig. 3.3.15). It was difficult to extract the cortex from the endospore-like structure of *Rba. johrii* hence the *Bacillus* cortex was used for the enzyme assay. When the vegetative cell walls (peptidoglycan) from *Bacillus* used as substrate for SleR protein, there was no OD loss observed therefore does not cleave the normal cell wall (Fig.

3.3.16). The enzyme assay indicates the SleR involves in cleaving the cortex of spore, it was annotated to be a cell wall hydrolase which may have role in cell wall cleavage during cell growth in cell division (during log phase). *In silico* study and enzyme assay indicates its properties of spore cortex lysis enzyme like SleB, which is expressed in spore formation stage (during stationary phase). To clear this real time expression analysis was done of *sleR* in *Rba. johrii* during its growth phases (Fig. 3.4.2). The expression of *sleR* was negligible between lag to stationary phase, but there was drastic increment in late stationary phase. This result was correlated with the differentiation of the cells parallelly where it was observed when the *sleR* expression was less no spores were observed but when the expression was increased the spores were found to be present. This pattern indicates that SleR protein does not participate in the development phases like in the peptidoglycan remodeling for cell growth and division (seen during the log phase). This higher expression of SleR at late stationary phase is similar to SleB which is also higher at the endospore formation stages (Boland *et al.,* 2000; Moriyama *et al.,* 1999; Ishikawa *et al.,* 1998).

Expression study strongly suggests that SleR is associated with the endospore like refractive structure formation during late stages and not in cell division at early stages of growth phases therefore the SleR protein has no role in peptidoglycan remodeling. To find out the expression is due to stress, we have taken two culture one with heat stress and another with normally grown *Rba. johrii* expression in these two conditions, the heat stressed culture has considerably bit higher than normal one grown in late stationary phase, therefore this suggest that heat stress does not increase the expression of SleR rather the endospore like structure which is formed due to heat stress (Fig. 3.4.2). Also, in late decline phase, the expression levels fall down dramatically suggesting that SleR is probably expressed in endospore like dormant

structure cells of *Rba. johrii* that are present in late stationary phase. Once sporulation is completed, there is no longer a requirement for SleR which may be why its expression decreased after the late stationary phase.

All the results show the similar properties to SleB which suggest that SleR is spore cortex lytic protein associated with the *Rba. johrii* endospore like structures. All these evidences with genome mining, draws a model for endospore like refractive structure formation in which there is not much similarity and differences with that of *Bacillus* spp. In a proteobacterium, *Myxococcus xanthus* forms fruiting bodies (spore like structure), in which a protein CbgA likewise was identified on the basis of sequence similarity (57% similarity and 35% identity) to SpoVR protein of *B. subtilis* known to have a function of spore cortex formation (Beall and Moran, 1994). Identified protein CbgA when characterized in *M. xanthus* has similar role in the cortex formation (Tengra *et al.,* 2006). Along with this some new genes related to fruiting body formation were identified by transcriptomic study one of the examples are a locus having 8 genes *nfsA-H* genes in *M. xanthus* (Muller *et al.,* 2010). In this organism fruiting spore bodies formation mechanism has few homologs of *B. subtilis* and few new genes identified. Here we have demonstrated experimentally along with the similarity-based identification that SleR has spore cortex lytic properties further new genes yet to be discovered for sporulation in *Rba. johrii*. This study was restricted to only *Rba. johrii* only and other Rhodobacteraceae members remains to be screen.

Taken together from all our results, we have consistently found similar functional properties of SleR with that of SleB. Therefore, our findings suggest that SleR, likely to be a spore cortex lytic protein which may be associated with the endospore like refractive structures of *Rba. johrii*. This evidence along with the inputs of genome mining project a model for

formation of endospores in *Rba. johrii* which shows many, if not all similarities with the model for *Bacillus* spp. Whether such structures are found in other Rhodobacteraceae members remains to be addressed as this study was limited to *Rba. johrii* and a few other members of the genus *Rhodobacter*. It will be intriguing if similar approaches could be taken to investigate these members and look into the molecular evidences to provide clues that can add to the *Rba. johrii* model of forming endospore like dormant structures.

In summary, the entire analysis advances the knowledge through evidences for the presence of "endospores" in *Rba. johrii*. The evidences include the studies from the staining analysis, ultrastructure analysis, and identification of putative homologues of protein encoding genes involved in *Bacillus* sporogenesis and from the characterized the novel SleR protein. Further, the study supports our previous findings (Girija *et al*., 2010) and has given leads into the sporulation of *Rba. johrii.* Therefore, a confined study based on ultra-microscopic and incomplete genome analysis concluding that "members of the phylum Proteobacteria do not produce endospores" (Beskrovnaya *et al.*, 2020) is rather an early assumption.

SUMMARY

5. Summary

The study was conducted with an aim to explore the insights of sporulation in a proteobacterium *Rba. johrii*. Spore like refractile bodies, malachite green, DAPI staining and TEM sectioning confirms the reproducibility of endospore like structure in *Rba. johrii.* The inhibition of PHA granules in *Rba. johrii* by acrylic acid and pentenoic acid clearly differentiated between PHA granules and spore like refractile bodies. The ultrafine structure of spore like refractile bodies showed similarity to *Bacillus* endospores with some distinction. A novel spore specific fluorescent stain "sporotan" was identified and staining method was developed to stain specifically the cryptic endospore like refractile bodies in *Rba. johrii.* The sporotan staining method is very selective, simple and efficient, which will help in the discovery of endospores produced by different members, also outside the phylum Firmicutes. The discovery of endospore like refractive structures in *Rhodobacter alkalitolerans* indicates the wide spread of these structures among other taxa of Rhodobacteraceae and probably among the phylum Proteobacteria.

One hundred protein homologs involved in *Bacillus subtilis* sporulation were identified in *Rba. johrii* genome including the signature proteins sigE, sigH, sig F, sig K, spo0A, spoIIAB, spoIIE, SpoIVB, sigG, spoIID, spoIIM, spoIVA, spoVB, spoVG ,yqfc, ytvI, sleB, SpoIVB, spoVAD, spoIIG and dapG. The signaling cascade protein homologs which has role in triggering spore initiation were also found to be conserved in *Rba. johrii* which indicates that there might be a similar spore initiation mechanism. Some of the spore associated proteins like "Small Acid Soluble Proteins (SASPs)", Dipicolinate (DPA) synthetase were not found in *Rba. johrii.* Putative spore cortex lytic protein having 28.4% amino acid identity with 67% protein coverage contains Hydrolse_2 domain was identified in *Rba. johrii*. Predicted Sle structure has seven -α-

helix, three -β-sheets, eleven loops and turns. From the alignment and superimposition, the catalytic residue Glu128 and alternate second Glu132 is conserved in Sle of *Rba. johrii* which is common in of SleB- type enzymes. Catalytic groove is lined with aromatic residues Phe167, Phe209, Trp218, His231, Phe233 which is conserved in all SleB and this is responsible for binding and cleavage of spore cortex peptidoglycan and binding with substrates is confirmed by docking.

In vitro enzyme assay for purified SleR protein cleaves spore cortex peptidoglycan (sacculi) as substrate but do not cleave the cell wall peptidoglycan. Expression pattern of SleR protein was low till the stationary phase, significant increase was found in the late stationary phase this expression pattern can be correlated with the formation of spore like structure. The characterization of putative Sle protein of *Rba. johrii* showed properties similar to spore cortex lytic enzyme SleB therefore we propose the name SleR for this putative protein. This novel spore cortex lytic enzyme (SleR) was characterized *in silico, in vitro* and its differential expression pattern confirmed in *Rba. johrii*.

Overall the work represents a gateway to the study of endospore-like structure formation in a non-Firmicutes member like *Rba. johrii* belonging to the phylum Proteobacteria. This work is a valuable contribution which opens scope in understanding the sporulation strategies adapted among the members of the phylum Proteobacteria which is far different from the members of Firmicutes.

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PUBLICATIONS

7. Publications

1. **Ali, Ashif**, Kumar Gaurav, N. Senthilnathan, T. P. Radhakrishnan, Sasikala Ch, and Ch V. Ramana (2020) "Sporotan" a new fluorescent stain for identifying cryptic spores of *Rhodobacter johrii*. *J Microbiol Methods* 177: 106019.

2. Suresh, G., B. Sailaja, **A. Ashif,** Bharti P. Dave, Ch Sasikala, and Ch V. Ramana. (2017) Description of *Rhodobacter azollae* sp. nov. and *Rhodobacter lacus* sp. nov. *Int J Syst Evol Microbiol* 67: 3289-3295.

Abstract and Conferences:

1. Poster presentation title **"Identification and *In silico* characterization of a putative spore cortex lytic enzyme (sleb) from *Rhodobacter johrii,* an endospore producing bacteria". Ashif Ali**, Ch. Sasikala and Ch.V.Ramana. International Conference on Innovations in Pharma and Biopharma Industry (ICIPBI-2017), 20-22 December, Hyderabad, India.

2. Poster presentation title **"Genome Sequence of *Rhodobacter johrii* JA192". Ashif Ali,** Ch. Sasikala and Ch.V.Ramana. The 8th international conference on photosynthesis and hydrogen energy research for sustainability- 2017, October 30-November 03, Hyderabad, India.

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"Sporotan" a new fluorescent stain for identifying cryptic spores of *Rhodobacter johrii*

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ABSTRACT

We propose a new fluorescent stain "sporotan" and staining protocol which aid in the identification of cryptic endospores which are otherwise mistaken as poly-β-hydroxyalkanoate granules.

1. Introduction

A spore is the tough, dormant, non-reproductive structure usually formed when bacteria go through extreme adverse environmental conditions. This is a survival strategy that evolves among many bacteria which are human pathogens such as *Bacillus anthracis, Bacillus cereus, Bacillus thuringiensis, Clostridium botulinum, Clostridium tetani* (Bottone, 2010). However, spores are major contaminants in food processing industry (Heyndrickx, 2011). Spores are also advantageous for humans as potential probiotics (Elshaghabee et al., 2017) because they are heat, acid, bile salt and gastric juice resistant, which are generally used for their antimicrobial, anti-oxidant, immune-modulatory and pathogen exclusion properties (Shobharani et al., 2015; Ripert et al., 2016; Lefevre et al., 2015).

Bacteria produce spores either endogenously (endospores) or exogenously (exospores). It is widely believed that endospore producers are confined to the members of the phylum *Firmicutes*. Other endospore producers discovered include *Rhodobacter johrii* (Girija et al., 2010), *Serratia marcescens* subsp. *sakuensis* (Ajithkumar et al., 2003), *Rhodopseudomonas palustris* (Venkidusamy and Megharaj, 2016), *Coxiella burnetii* (McCaul and Williams, 1981) and *Myxococcus xanthus* (Kroos, 2007; Kaiser, 2006) belonging to the phylum Proteobacteria. *Mycobacterium* species like *Mycobacterium marinum*, *Mycobacterium bovis BCG* (Ghosh et al., 2009) and *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Lamont et al., 2012) belong to the phylum *Actinobacteria,* indicating clearly that the phenomenon of endospores is wide spread in bacteria and not confined to the members of the phylum Firmicutes, as believed previously (Abecasis et al., 2013). On the other hand, exospore producing bacteria are wide spread among the members of the phylum Actinobacteria (Reed and Dugan, 1979; Reed et al., 1980) and a few members of Proteobacteria (Kimble-Long and Madigan, 2001; Wu et al., 2005). Detection of bacterial spores is an important part of medical science, as well as food, bio-fertilizers and pharma industry. Therefore, an easy, rapid method for their visualization and identification is required, as the existing spore staining methods have various drawbacks.

The classical methods for endospore staining are Gram stain and Schaeffer–Fulton method (Schaeffer and Fulton, 1933) which was modified from Dorner's method (Dorner, 1922) which is lengthy, tedious and hazardous. The recent method of "Flood to microliter" developed by Siguenza and coworkers (Siguenza et al., 2019) for bacterial endospore staining utilizes microliters of the dye, still flooding of dyes is practiced. There are a large number of fluorescence dyes also used for endospore staining, however, they all still have several drawbacks (Table.1). Some of these like 4–6-diamino-2- phenylindole [DAPI] and propidium monoazide [PMA] cause impairment of living cells and are only permeable in membrane–compromised dead cells; thus, they bind to both vegetative cells as well as dead spores. Acridine orange [AO], DAPI, PMA and flow cytometery fluorescent probes like, SYBR1, SYBR2 (Invitrogen™) are not specific for binding spore DNA (Schichnes et al., 2006; Tarnowski et al., 1991; Probst et al., 2012; Karava et al., 2019). Auromine 0, Thioflavin T [ThT] and Hoechst 34580 exhibit binding for both vegetative cells and spores (Bartholomew et al., 1965; Xia et al., 2011; D'Incecco et al., 2018). We propose a new fluorescent bacterial spore stain "Sporotan" which is chemically: 7,7-(*n*-hexylamino)-8,8-

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Description of *Rhodobacter azollae* sp. nov. and *Rhodobacter lacus* sp. nov.

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Abstract

Three strains (JA826^T, JA912^T and JA913), which were yellowish brown colour, rod to oval shaped, Gram-stain-negative, motile, phototrophic bacteria with a vesicular architecture of intracytoplasmic membranes, were isolated from different pond samples. The DNA G+C content of the three strains was between 64.6 and 65.5 mol%. The highest 16S rRNA gene sequence similarity of all three strains was with the type strains of the genus *Rhodobacter sensu stricto* in the family *Rhodobacteraceae*. Strain JA826^T had highest sequence similarity with *Rhodobacter maris* JA276^T (98.5 %), *Rhodobacter viridis* JA737^T (97.5 %) and other members of the genus *Rhodobacter* (<97 %). Strain JA912^T had highest sequence similarity with *Rhodobacter viridis* JA737^T (99.6 %), *Rhodobacter sediminis* N1^T (99.3 %), *Rhodobacter capsulatus* ATCC 11166^T (98.8 %) and less than 97 % similarity with other members of the genus *Rhodobacter*. The 16S rRNA gene sequence similarity between strains JA826^T and JA912^T was 96.9 %. DNA–DNA hybridization showed that strains JA826^T and JA912^T (values among themselves and between the type strains of nearest members <44 %) did not belong to any of the nearest species of the genus *Rhodobacter*. However, strains JA912^T and JA913 were closely related (DNA–DNA hybridization value >90 %). The genomic distinction was also supported by differences in phenotypic and chemotaxonomic characteristics in order to propose strains JA826^T (=KCTC 15478^T=LMG 28758^T) and JA912^T (=KCTC 15475^T=LMG 28748^T) as new species in the genus *Rhodobacter sensu stricto* with the names *Rhodobacter lacus* and *Rhodobacter azollae*, respectively.

The genus *Rhodobacter* (*Rba*), proposed by Imhoff *et al.* [1] upon reclassification of the species of the genus *Rhodopseudomonas*, accommodated a few species of purple nonsulfur anoxygenic phototrophic bacteria namely, *Rhodobacter capsulatus* (type species), *Rhodobacter sphaeroides*, *Rhodobacter sulfidophilus* and *Rhodobacter adriaticus,* with a vesicular architecture of intracytoplasmic membranes. However, *Rba. sulfidophilus* and *Rba. adriaticus* were subsequently reclassified as *Rhodovulum sulfidophilum* and *Rhodovulum adriaticum*, respectively [2]. At the time of writing, the genus *Rhodobacter* comprises 13 recognized species names: *Rhodobacter aestuarii*, *Rhodobacter azotoformans*, *Rhodobacter blasticus*, *Rhodobacter capsulatus*, *Rhodobacter johrii*, *Rhodobacter maris*, *Rhodobacter megalophilus*, *Rhodobacter ovatus*, *Rhodobacter sphaeroides*, *Rhodobacter veldkampii*, *Rhodobacter vinaykumarii*, *Rhodobacter viridis* and the recently described *Rhodobacter sediminis* [3]. The genus *Rhodobacter* in the family *Rhodobacteraceae* includes a very heterogeneous assemblage of phototrophic bacteria with a large number of interspersing chemotrophic bacteria and their evolutionary relationships are not well established. According to the current taxonomy based on 16S rRNA gene sequence phylogenetic analysis, *Rhodobacter* species are grouped into five monophyletic clusters, each of which comprise one to seven species. Here, we propose to include the description of two new species of phototrophic bacteria affiliated to the genus *Rhodobacter sensu stricto*.

Strain JA826^T was isolated from a water sample of an industrially polluted fresh water lake near Hubbali, Karnataka, India (75° 20′ E, 15° 26′ N; sample pH 6.8). Strains JA912^T and JA913 were isolated from a fern, *Azolla filiculoides*, growing in a fresh water pond in Gujarat, India (GPS position: 72° 43′ E, 22° 47′ N; sample pH 6.0 and salinity 0.8 %). Both samples were serially diluted (10-fold dilution) and pour plated by using the wax overlay method [4] and incubated at 30 °C in the

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains JA826^T, JA912^T, JA913 are LN835251, LN810641 and LN810642, respectively
Six supplementary figures and one supplementary table are available with the online Supplementary Material.

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