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RESEARCH ARTICLE

Molecular and Genomic Characterization of PFAB2: A Non-virulent *Bacillus anthracis* Strain Isolated from an Indian Hot Spring

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Abstract: ***Background*:** Thermophilic bacilli in both aerobic or facultative anaerobic forms have been isolated for over a hundred years from different mesophilic or thermophilic environments as they are potential source of bioactive secondary metabolites. But the taxonomic resolution in the *Bacillus* genus at species or at strain level is very challenging for the insufficient divergence of the 16S rRNA genes. One such recurring problem is among *Bacillus anthracis*, *B. cereus* and *B. thuringiensis*. The disease-causing *B. anthracis* strains have their characteristic virulence factors coded in two well-known plasmids, namely pXO1 (toxin genes) and pXO2 (capsule genes).

***Objective*:** The present study aimed at the molecular and genomic characterization of a recently reported thermophilic and environmental isolate of *B. anthracis*, strain PFAB2.

***Methods*:** We performed comparative genomics between the PFAB2 genome and different strains of *B. anthracis*, along with closely related *B. cereus* strains.

***Results*:** The pangenomic analysis suggests that the PFAB2 genome harbors no complete prophage genes. Cluster analysis of Bray-Kurtis similarity resemblance matrix revealed that gene content of PFAB2 is more closely related to other environmental strains of *B. anthracis*. The secretome analysis and the *in vitro* and *in vivo* pathogenesis experiments corroborate the avirulent phenotype of this strain. The most probable explanation for this phenotype is the apparent absence of plasmids harboring genes for capsule biosynthesis and toxins secretion in the draft genome. Additional features of PFAB2 are good spore-forming and germinating capabilities and rapid replication ability.

***Conclusion*:** The high replication rate in a wide range of temperatures and culture media, the non-pathogenicity, the good spore forming capability and its genomic similarity to the Ames strain together make PFAB2 an interesting model strain for the study of the pathogenic evolution of *B. anthracis*.

Keywords: *Bacillus anthracis*, avirulence, comparative genomics, pangenomics, pathogenesis, secretome analysis.

1. INTRODUCTION

Thermophilic bacilli in both aerobic or facultative anaerobic forms have been isolated for over a hundred years from different mesophilic or thermophilic environments. Importance of thermophilic bacilli is increasing as they are a potential source of thermostable enzymes, pigments or polysaccharides [1]. Established by Cohn in 1872, the genus *Bacillus* has gone through several taxonomic changes. As sequence analysis continues to lead to the separation of groups of species from the core genus *Bacillus* to form new genera, the definition of novel genera also changes to allocate new isolates [2]. The insufficient divergence of the 16S rRNA genes in the *Bacillus* genus makes the taxonomic resolution at species or at strain level very challenging. One such recurring problem is *Bacillus anthracis*, *B. cereus*, and *B. thuringiensis* are basically one species on the basis of genetic evidences [3]. The Gram-positive bacterium *Bacillus anthracis* is a major concern for human and animal health due to its high lethality and to the difficulty in developing safe and effective preventive and therapeutic strategies against it. Even though a number of physical and chemical agents were shown to inactivate *B. anthracis* spores [4, 5], there is still much concern about the possible acquisition of pathogenicity by non-virulent spore-forming *B. anthracis* variants through plasmid DNA transfer. The disease-causing *B. anthracis* strains have their virulence factors coded on two well-known plasmids, namely pXO1 (toxin genes) and pXO2 (capsule

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genes). A strain lacking one of these plasmids is regarded as a low-virulence or attenuated strain, and the Sterne strain, which lacks plasmid pXO2, is one such example.

However, phylogenomic analyses suggest that *B. anthracis* evolved from a free-soil bacterium to an obligate pathogen [6]. Particularly, the gain of genes from extrachromosomal DNA has made *B. anthracis* distinct from its two close relatives, *viz*. *B. cereus* and *B. thuringiensis* [6]. Though there have been several reports on pathogenic *B. anthracis* that discuss the characteristic genetic features of this anthrax bacterium [7-9]; environmental isolates with close genomic identity with *B. anthracis* Ames strain had been proven to be avirulent *B. anthracis* to date [7]. Although few environmental strains that closely resemble *B. anthracis* were non-pathogenic but later they were found to be a member of the *B. cereus* group during the investigation [10, 11].

In NCBI GenBank database, genome assembly of 1041 *B. cereus*, 513 *B. thuringiensis* and 233 *B. anthracis* are available (Accessed on: 17.06. 2019). Considering the complexity of this group, the description of a new isolate requires a broad comparative study to the known strains. Here, we describe the analysis of a new thermophilic *Bacillus* isolate, namely strain PFAB2, using a pangenomic approach. The term "pangenome" reflects the total number of non-redundant genes in a dataset and is mainly divided into two parts; *viz*. the core genome and the accessory genome. While the core genome is composed of genes shared among all genomes and are mostly involved in essential cellular processes, the accessory genome is formed by dispensable and unique genes, which are specific to a few or to a single strain, respectively, and are mainly involved in niche adaptation [12]. Furthermore, we performed *in vitro* and *in vivo* assays to get a broader idea on the gene composition and phenotypic behavior of the PFAB2 strain. For a better understanding on both pathogenesis/virulence and evolutionary lineages of *B. anthracis* from respectively harmless *B. cereus* to virulent *B. anthracis*, it is important to identify the unique environmental origin *B. anthracis* strains from extreme habitats, a potential missing link. Thus, our pangenomic approach may bring new insights on evolution of *B. anthracis* genetic diversity and niche adaptation.

Hence, the present study aimed at characterizing a recently reported thermophilic and environmental variant of *B. anthracis*, strain PFAB2 [13, 14]. We performed a comparative genomic study between strain PFAB2 with other environmental and pathogenic strains of *B. anthracis* along with the closely related *B. cereus* strains. Unique features of this environmental strain were also unwrapped with the help of several *in vitro* as well as *in vivo* experiments to determine the unknown and unique attributes of the concerned strain.

2. METHODS

2.1. Sampling and Collection

Strain PFAB2 is an avirulent variant of *Bacillus anthracis* isolated from hot spring water of Panifala, West Bengal (India) in a previous study by our group [13]. This strain grows well in Nutrient Broth (NB) medium at 45ºC. Methods of the entire study are elucidated in a flowchart as Supplementary Fig. (**1**).

2.2. Bacteriological Analysis

Preliminary morphological analysis of PFAB2 was performed using a Leica DM1000 fluorescence microscope at 100× magnification by incubating the isolate in 1 mM zinc acetate solution followed by binding with a Zn ligand [15]. Morphological and physiological characterization was performed according to the standard protocols. Different biochemical characteristics of the bacterial isolate PFAB2, such as enzyme activity (catalase, amylase, protease, lipase) substrate hydrolysis, ability to produce gas, utilization of different carbon sources, salt tolerance, temperature, pH, motility, hemolysis and thermal death point were tested by following standard methods [16]. The susceptibility of PFAB2 to antibiotics was tested using agar diffusion assay with commercial antibiotic discs (Himedia) [17].

2.3. Genomic DNA Isolation and Library Construction

Isolation of genomic DNA, library construction and sequencing had been earlier reported by Banerjee *et al*. [13]. Plasmid DNA isolation was performed according to a Miksell *et al*. [18]. Average nucleotide identity (ANI) of PFAB2 was calculated based on the BLAST algorithm ANIb [19, 20] with recommended 95% species cut-off and higher than 0.99 for the TETRA signature [21].

2.4. Dataset Preparation for Comparative Genomics Analysis

NCBI GenBank database was used to prepare the dataset for comparative *in silico* study. We selected all the environmental *B. anthracis* strains, pathogenic *B. anthracis* type strains and closely related *B. cereus* strains over a total 233 *B. anthracis* and 1,009 *B. cereus* genome sequences deposited in the GenBank database as on August 30th, 2018. This subdataset comprised a total 15 *Bacillus* complete genomes of which 8 are from *B. anthracis* and 7 are from *B. cereus* strains. Further comparative *in silico* analysis and pangenomic study investigated the genetic basis of the putative avirulent phenotype exhibited by *B. anthracis* PFAB2.

2.5. Determination of OrthoANI and Heat Map Study

To establish the relatedness among the different strains, Average Nucleotide Identity (ANI) analysis was performed using OAT tool (Orthologous Average Nucleotide Identity Tool). OAT uses OrthoANI to evaluate the overall similarity between two genome sequences. Unlike the original ANI algorithm, OrthoANI results into identical reciprocal similarities. For both OrthoANI and original ANI, the proposed cut-off is 95-96% for species delineation [22]. An ANI heat map was prepared using OAT to understand whether *B. anthracis* PFAB2 is more closely related to *B. anthracis* or to *B. cereus*.

2.6. Subsystem Gene Categorization, Identification of Unique Genes and Cluster Analysis

An open source prokaryotic genome annotation system Rapid Annotation using Subsystem Technology (RAST) [23] was used for subsystem assignment to *B. anthracis* and *B. cereus* genomic sequences of the dataset. Functionally annotated genomes were visualized using SEED Viewer and the subsystem features were compared in the entire dataset.

We integrated and analyzed the presence of subsystem genes using Primer-6 (Primer-E) software. The similarity in the gene content among the dataset was analyzed by constructing a Bray-Curtis similarity matrix. Non-metric multidimensional scaling (nMDS) was used to build a constrained ordination of the samples based on gene content.

2.7. Pan, Core and Accessory Genome Characteristics Determination

Pangenomes were formed to understand and estimate the number of shared genes (core genome) and unique/variable genes (accessory genome) using Spine and AGEnt tool [24]. Spine, a Perl-based program was used to determine the core genome from the selected genomic sequences. Only alignments with ≥ 85% sequence identity were considered homologous [20]. Prediction of accessory genome in bacterial genomic sequences was performed using AGEnt algorithm that depends on a combination of the NUCmer function of the MUMmer software package v3.23 and perl script. An individual gene was considered to belong to accessory genome if ≥ 50% of its sequence was located within the coordinates of an accessory region (*i.e.* unique strain-specific region) [24]. Additionally, the entire genome set (Pan genes), each core genome and unique accessory genomes had been functionally characterized using RAST tool.

The interrelationship amongst pangenome, PFAB2 core genome and accessory genome was analysed using genevenn [25] and also classified to Clusters of Orthologous Groups (COG) family by probing against the COG database using online WebMGA tool [26]. Final subelement gene set (Common longest nucleotide stretch in all the strains) was subjected for WebMGA analysis for COG percent categorization. PHASTER (PHAge Search Tool Enhanced Release), an upgraded version of PHAST web server was used [27] for the rapid identification and annotation of prophage sequences within the *B. anthracis* and *B. cereus* core and accessory genomes of the dataset.

2.8. Study of Growth Curve and Thermotolerance

Growth of *B. anthracis* PFAB2 was measured at 37ºC, 45ºC, 50ºC and 55ºC and compared to that of *B. anthracis* strain Sterne. For this study, both strains were incubated in Brain Heart Infusion (BHI) media at 120 rpm and under constant 5% CO_2 supply. At every hour, the optical density was measured at appropriate dilution up to the stationary phase of bacterial growth (Supplementary information). The thermotolerance of PFAB2 was determined by growing the strain at higher temperatures from 37-55ºC.

2.9. Biofilm and Pellicle Formation Study

Biofilm was developed by growing bacterial cells under static condition as previously reported [28]. Pellicle was allowed to form in culture tubes under static conditions. *B. anthracis* cells in logarithmic phase cultured in BHI medium were used as inoculums for both biofilm and pellicle formation study. Pellicle cells were visualized by direct observation after its maturity and biofilms formed under different temperatures were quantitatively calculated by crystal violet staining as reported previously [28-30] and qualitatively by cell morphology analysis using DAPI staining followed by confocal microscopic observations with Olympus FluoView™ FV1000.

2.10. Protein and Toxin Estimation in the Secretome

B. anthracis produces its principal virulence factors Protective Antigen (PA), Lethal Factor (LF) and Edema Factor (EF) in the medium [30, 31]. It also acquires *B. anthracis*-specific characteristic novel raft marker FlotP protein having no virulent effects which is secreted in the medium [32]. To understand cytotoxicity associated with the toxins released in culture media supernatant, RAW 264.7 macrophage cells were incubated with BHI media supernatants. For this, *B. anthracis* PFAB2 and control strain Sterne were allowed to grow overnight at 37ºC in BHI media. Culture was centrifuged at 10,000 × g for 15 min at 4ºC and supernatant was collected and filtered using 0.22 µm filter (MDI). The filtrate was then concentrated about 5 times using rotary evaporator and was used for cytotoxicity assay and protein estimation.

Amount of toxins secreted under different stress conditions was quantified using ELISA. Briefly, the media supernatants from BHI grown cultures were concentrated ~8 times in rotary evaporator. Concentrated retentate containing proteins were collected and were used for quantification study. A 96-well microtitre plate was coated with the retentate and incubated for 16 h at 4ºC. Purified rPA, rLF, rEF toxins were used as a positive control to prepare the standard curve [30, 33-35]. Plates were then blocked using 2% BSA for 1 h at room temperature (RT). Three successive washings were done by using 1× PBST containing 0.05% Tween-20. Plates were then incubated for 1 h at RT with monoclonal goat anti-PA, anti-LF and anti EF antibodies respectively (771B, 772B, 773B; List Biologicals respectively). After three washes with PBST, the plates were incubated for 1 h at RT with antigoat-IgG-HRP (1: 10,000) dilution. The plates were then washed and allowed to react with TMB substrate (BD Pharmingen). Finally, the OD was measured at 630 nm.

Cytotoxicity assay was performed by 5-fold concentration of the media supernatant from all the strains. For this, grown RAW 264.7 cells (2.5×10^4) had been seeded in 96-well microtitre dish for 16 h. The cells were then incubated with different dilutions of concentrated and filtered culture supernatants at 37ºC in humidified 5% CO_2 for 6 h. 1 µg/ml purified lethal toxin was taken as positive control [30]. Cells were incubated with MTT dye (dissolved in serum-free DMEM at a concentration of 5 µg/ml) for 1 h at 37 ºC to allow the dye uptake and oxidation of the dye by viable cells to analyze the percent viability. The medium was then replaced using DMSO and again incubated for 15 min with shaking to dissolve the insoluble formazan crystals. Finally, cell survival was calculated by measuring the absorbance at 540 nm.

2.11. *In vitro* and *In vivo* Pathogenesis Study

For *in vitro* study, mouse macrophage cell line, RAW 264.7 was maintained in DMEM (Sigma) with 10% heat inactivated fetal bovine serum (Gibco) containing appropriate concentrations of penicillin, streptomycin and amphotericin B (HiMedia). For experiment, cells were seeded in serum and antibiotic free DMEM, at a density of 2×10^6 cells per well in 6 well dishes and incubated overnight in a humidified

5% CO_2 condition. Infection with vegetative bacilli of both strain PFAB2 and control strain Sterne had been done according to Russell *et al*. [36]. Briefly, midlog phase bacilli from BHI media was washed with serum and antibiotic free DMEM, and allowed to infect macrophage cells for 1 h. The media containing unbound bacilli had been removed and washed twice with 1× PBS. To study bacterial association (extracellular and intracellular), the cells were lysed with chilled PBS and the dilutions were plated on BHI agar. To assess bacterial internalization, washed macrophages were further incubated with incomplete DMEM with gentamicin (20 µg/ml) for 1 h at 37ºC/5% CO_2. Cells were washed again followed by lysis with chilled 1× PBS. Appropriate dilutions of lysate were plated on BHI agar for CFU count.

For *in vivo* pathogenesis, six to eight weeks young male Swiss-Albino mice were challenged intraperitoneally (i.p.) with 2×10^4 *B. anthracis* grown under BHI media, suspended in PBS. 8 mice for each set of PFAB2 and Sterne are observed for mortality study along with control mice for 10 days. The death curve of the mortality study was plotted according to the Kepler-Miller method [30].

2.12. Spore Formation and Germination Study

B. anthracis spores were prepared by inoculating 10% of overnight grown *B. anthracis* PFAB2 culture in sporulation broth (HiMedia). Spore formation capacity was compared to control strain Sterne. The culture was kept at 37ºC, 120 rpm shaking for 5 days which was then subjected to heating at 60ºC for 60 min to kill all the vegetative cells. Spores were centrifuged and the pellet was washed with autoclaved MQ water. Spore concentration was calculated by serial dilution method. 5×10^4 spores were inoculated into fresh BHI broth and were grown at 37ºC. Each aliquot was taken after every 5 min till 30 min and heat treatment was given at 65ºC for 30 min to kill germinated spores or vegetative bacilli. Germination efficiency was determined by calculating the CFU/ml obtained after plating the samples in different dilutions. For imaging the spores of *B. anthracis* PFAB2, transmission electron microscopic analysis was done where freshly overnight grown samples were fixed on carbon coated grids and stained with 0.2% uranyl acetate to observe under JEM-1011 100KV transmission electron microscope.

3. RESULTS AND DISCUSSION

3.1. Bacteriological Analysis

Several cells of isolate PFAB2 were observed in usual dividing conditions like other firmicutes with fluorescence microscopic analysis. The average minimum size of each bacilli was found to be ~4.2 µm in length as depicted in Fig. (**1**). Further analysis confirmed that PFAB2 is a Gram-positive, catalase and oxidase enzyme positive bacterium and it is halotolerant up to 6%. Optimum growth of the bacteria was found to be 45ºC. Its biochemical parameters are similar to those of *B. anthracis* including negative hemolysis and penicillin resistant nature (Supplementary Table **1**). Even the antibiotic sensitivity, motility and fermentation abilities also favor its similarity to *B. anthracis* among the three close *Bacillus* species; *viz*. *B. anthracis*, *B. cereus* and *B. thuringiensis*.

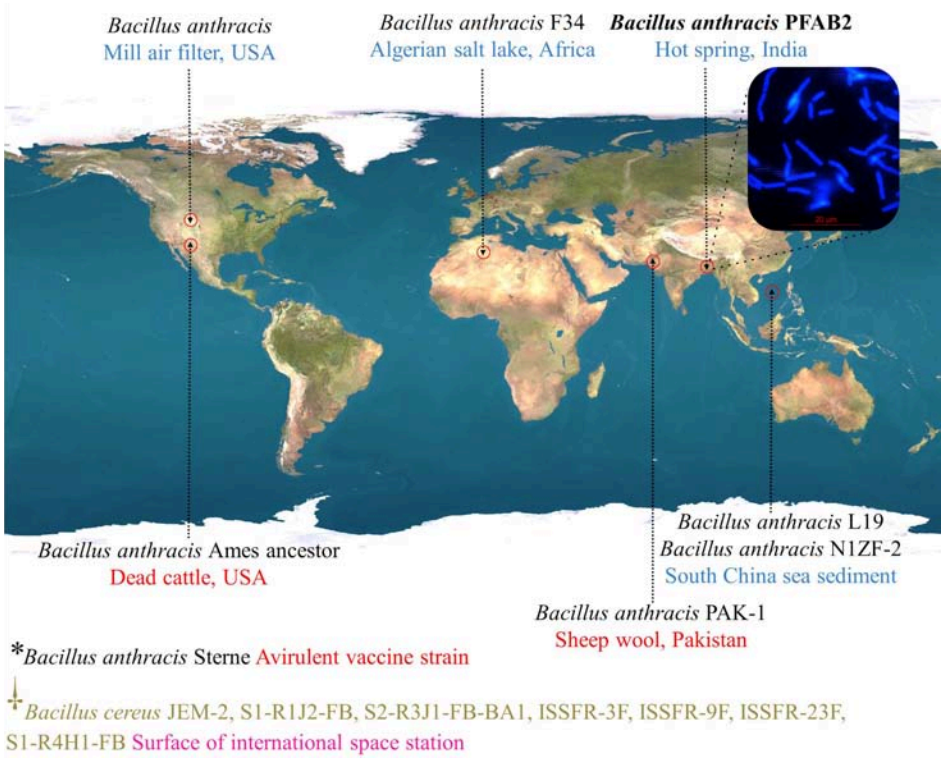


Fig. (1). Geographical distributions of the 15 *Bacillus* strains including environmental *B. anthracis* (on the inset image: fluorescence microscopy of *B. anthracis* PFAB2), pathogenic *B. anthracis* and related *B. cereus* strains. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.2. Whole Genome Sequencing and Dataset Preparation for Comparative Genomics Analysis

The draft genome sequence was already deposited in GenBank as *Bacillus anthracis* PFAB2 under the accession no. MEAQ00000000 [13]. Based on Average Nucleotide Identity (ANI) analysis (http://www.ezbiocloud.net/ezgenome/ani; Last accessed: 02.02.18), *B. anthracis* PFAB2 is 97.93% similar to reference strain *B. anthracis* Ames and 91.56% to *B. cereus* ATCC14579, confirming its species level identity to *B. anthracis.* It also showed closest tetranucleotide usage pattern value of 0.99957 with *B. anthracis* Ames Ancestor (http://jspecies.ribohost.com/jspeciesws/#analyse; Last accessed: 02.02.18). The recommended species cut-off is 95% for ANI indices and >0.99 for the TETRA signature [21]. The extracted 16S rRNA gene has shown 100% identity with *B. anthracis* ATCC 14578(T) followed by 99.84% similarity with *B. cereus* ATCC 14579(T). Interestingly and unlike *B. anthracis* Ames strain, both virulence plasmid (pXO1 and pXO2) were absent in PFAB2. *B. cereus*, *B. anthracis* and *B. thuringiensis* are highly inter-related species. For comparative genomics study, *B. thuringiensis* was excluded because of less than 90% ANI value and the total dataset comprises of different strains *B. anthracis* and *B. cereus*. As can be seen from Fig. (**1**), a total of 14 strains were selected based on maximum ANI similarity with *B. anthracis* PFAB2 which consists of reference *B. anthracis* strains like Ames Ancestor, Sterne, environmentally reported *B. anthracis* variants from sea sediments, air filter or salty lake, few close pathogenic variants and closely related *B. cereus* strains reported from international space station. Information regarding the source of the strains and the sequences was retrieved from NCBI GenBank database and had been tabulated in Supplementary Table **2**.

3.3. Determination of OrthoANI and Heat Map Study

Species demarcation in prokaryotes is principally based on overall genome relatedness. Recent research trend on the observation of genome relatedness between two strains is shifting from experimentally determined similarity obtained by DNA-DNA hybridization (DDH) to genome-sequence-based similarity. Another approach to understand species relatedness is sharing the orthologous genes. Identification of orthologs is important for steadfast prediction of gene function in newly sequenced genomes. In our study, OAT had been used for OrthoANI determination and heat map formation, for which genome sequences of the dataset were fragmented and only orthologous fragment pairs were taken into consideration for measuring nucleotide identities [22]. Considering both Fig. (**2A**) and Fig. (**2B**), it was observed that PFAB2 shared 98.2% orthologous common ancestral genes with three members of *B. cereus* family; *viz*., S2-R3J1-FB-BA1, S1-R4H1-FB and ISSFR-23F reported from international space station. Interestingly, PFAB2 also shared 98.1% orthologous genes with *B. anthracis* clade that consisted potentially virulent Ames Ancestor and PAK-1 strains. Another important finding was PFAB2 had relatively low orthoANI value with the environmental variants of *B. anthracis* reported from sea sediment or salt lake that reciprocated more species closeness of PFAB2 with environmental *B. anthracis*. Thus from the OrthoANI heat map, it may be perceived that PFAB2 is a unique *B. anthracis* variant which shares common ancestral genes with both pathogenic *B. anthracis* and avirulent *B. cereus* strains. Presence of relatively more common ancestral genes with *B. cereus* compared to *B. anthracis* hinted its evolution from avirulent *B. cereus* to *B. anthracis* and not the opposite. So *B. anthracis* PFAB2 may be substantially positioned as a separate branch point of anthrax evolution and can be a missing link between the *B. cereus* and *B. anthracis*.

3.4. Subsystem Gene Categorization, Identification of Unique Genes and Cluster Analysis

As strain PFAB2 shared common ancestral genes with both avirulent *B. cereus* and pathogenic *B. anthracis*, it was important to find its species uniqueness. It was observed from RAST analysis that PFAB2 genome consisted of *B. anthracis* specific sigma factor B, phospholipase C, internalin-like genes, germination genes (*ger*), iron acquisition genes; all of that had been earlier reported from the virulent *B. anthracis* strains too. Absence of immune inhibitor A metalloprotease indicated no virulence or insect-infecting lifestyle in recent ancestors. Phosphatidyl choline-preferring phospholipase C was also found to be absent which is otherwise a characteristic gene for pathogenic *B. anthracis* variants. It also featured additional UV-induced repair capacities through the presence of deoxyribodipyrimidine photolyase genes. Presence of thioredoxin, thioperoxidase, superoxide dismutase (Cu-Zn precursor) in PFAB2 indicated killing of nitric oxide-mediated macrophage cell lysis. But the most striking difference had been observed in the phage genes subsystem category. While there were ~37 phage genes present in reference Ames Ancestor strain, there were only ~5 phage genes present in PFAB2. PFAB2 had revealed its species uniqueness for consisting several *B. anthracis* specific genes along with absence of pathogenicity-related factors. *B. anthracis* PFAB2 was also earlier reported to be a unique non-capsulated *B. anthracis* variant as the entire poly-γ-*D*-glutamic acid biosynthesis pathway related genes were absent [13].

Cluster analysis of Bray-Kurtis similarity resemblance matrix on total subsystem category genes of all 15 strains clearly hinted more closeness of PFAB2 with other environmentally reported *B. anthracis* from mill air filter, sea sediments or salt lake; based on the gene contents. Entire subsystem category genes were resulted into two clusters where one cluster was dominated by environmentally reported *B. anthracis*, while the other by pathogenic type *B. anthracis* strains and *B. cereus* (Fig. **3**). More specifically, in terms most common subsystem genes present, *B. anthracis* PFAB2 was observed to be most similar to *B. anthracis* Mill air filter USA (Fig. **3**).

3.5. Pan, Core and Accessory Genome Characteristics Determination

The whole genome of *B. anthracis* give the hint of the presence of novel accessory genomic elements in each strain. Thus in order to get a probable conclusion on evolution of this unique strain PFAB2, pangenomic approach was taken. The algorithm AGEnt computationally subtracted previously derived core genome sequences from a query draft or complete genome sequence to result into the accessory genome.

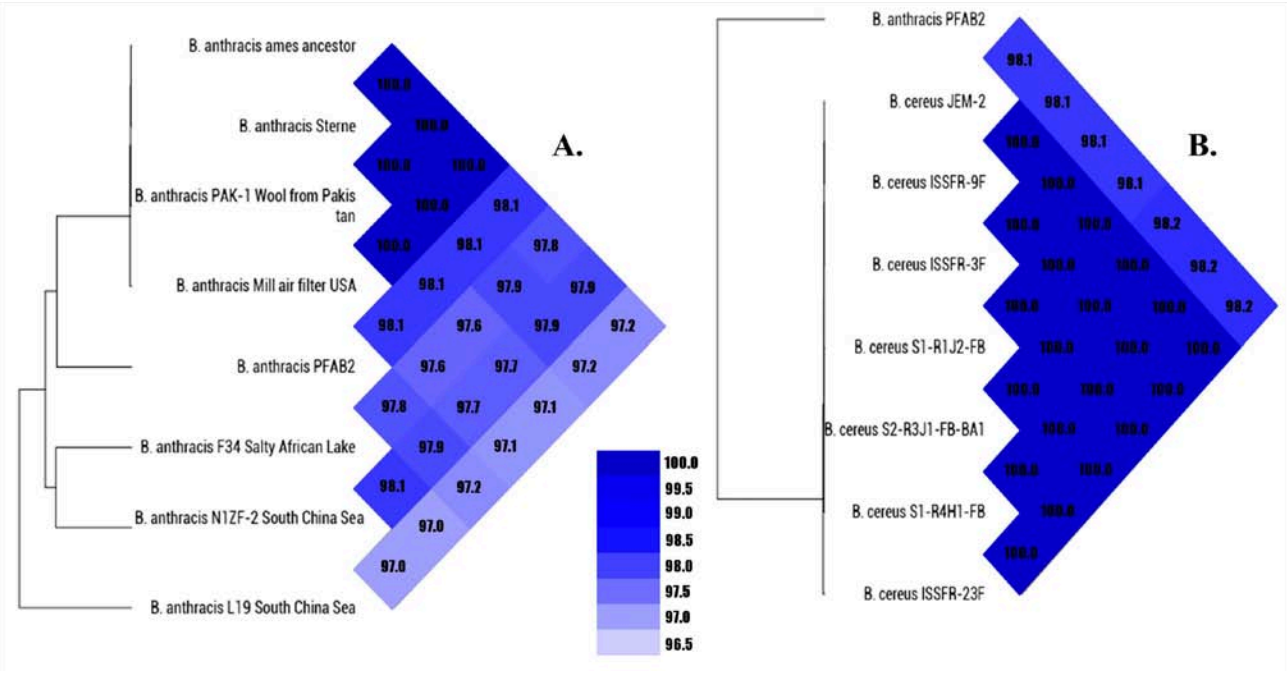


Fig. (2). OrthoANI heatmap of *B. anthracis* PFAB2 showing common ancestral relatedness with all pathogenic *B. anthracis*, environmentally reported *B. anthracis* and avirulent *B. cereus*. where **A.** comparison of orthologous average nucleotide identity of *B. anthracis* PFAB2 with other *B. anthracis* isolates, and **B.** comparison of orthologous average nucleotide identity of *B. anthracis* PFAB2 with related *B. cereus* isolates. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

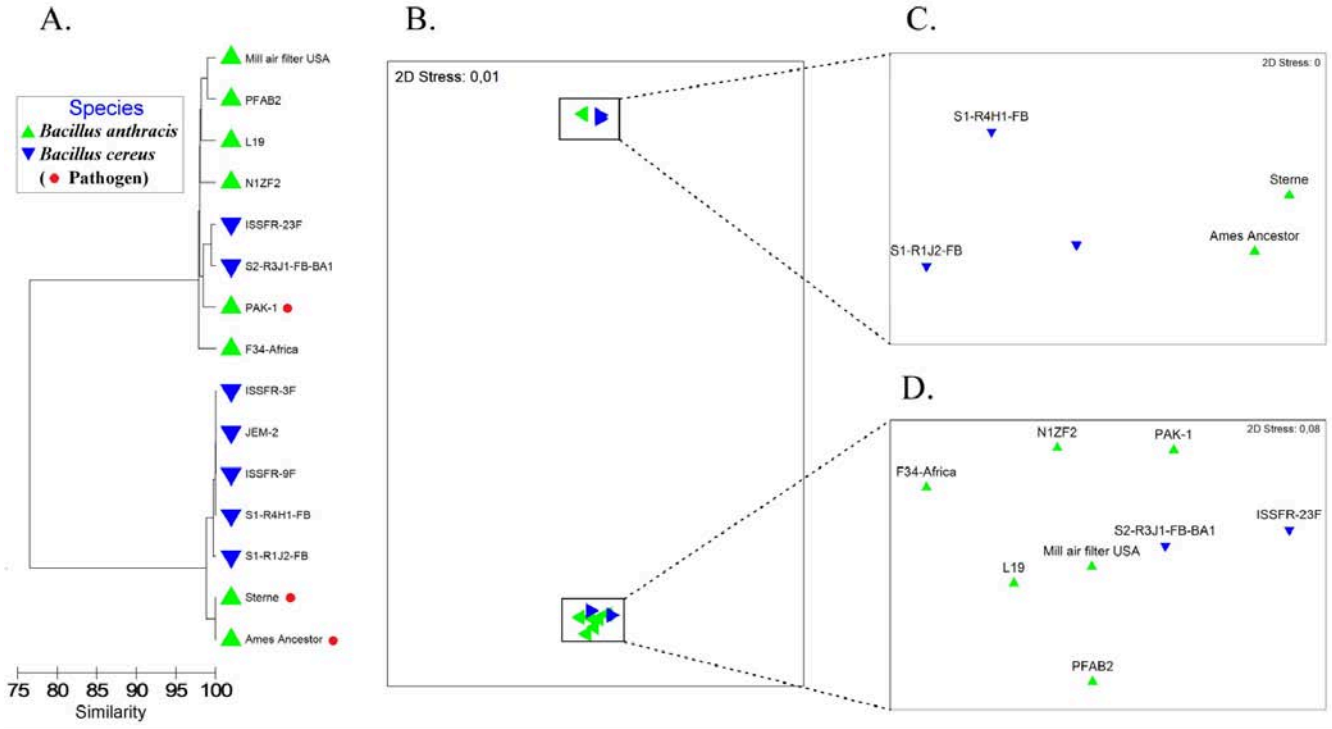


Fig. (3). Cluster analysis of Bray-Kurtis similarity resemblance matrix of the 15 *Bacillus* strains based on the presence of all subsystem genes; where **A**. tree showing the close lineage of *B. anthracis* PFAB2 with environmental *B. anthracis*; Mill air filter USA, **B**. nMDS 2D stress plot showing two main clusters among the 15 *Bacillus* strains, **C**. Cluster 1 mostly dominated by *B. cereus* and pathogenic type strains of *B. anthracis*, and **D**. Cluster 2 dominated by environmentally reported variant of *B. anthracis*, including PFAB2. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

The accessory genome was enriched with a number of mobile genetic elements. Following extraction of the core genome, the remaining sequences of the 15 reference strains were selected as the accessory genome.

The accessory regions were found to be rich in motility and chemotaxis related genes, prophage sequences, and disease and defense related genes as shown in Fig. (**4A**). Sharing of genes and interrelationship among the pan, PFAB2 core and PFAB2 accessory genome is depicted in Fig. (**4B**). The most striking difference was observed in case of phage genes. While almost no phage regions were present in core region, more than 15% of the total accessory region was composed of prophage sequences only. GC content of the accessory region (33.49%) was also lower than that of core genome (35.74%). The average size of the accessory genome was 1071 Kbp, representing 19.97% of the total genome in entire reference set. More specifically, accessory genome for *B*. *anthracis* constituted of 21.23% of the total genome; while for *B*. *cereus* isolated from international space station had a mean of 18.37% of total accessory genome. Importantly, accessory genomic region percentage of strain PFAB2 (17.61%) was found very close to that of Ames Ancestor strain (18.09%), followed by Sterne (18.11%) and not to that of *B*. *cereus*. The average size of unique region was comparatively much higher as identified through pangenomic analysis. In a recent study on *Pseudomonas aeruginosa*, the average accessory genomic region was identified as only 11.1% of the total genome [24]; whereas accessory genome for *B*. *anthracis* group constituted 21.23% of the total genome.

The minimum set of accessory genomic elements in the population is the subelement and it determines the distribution of each accessory genomic element among the genomes. The final subelement file obtained through ClustAGE analysis was distributed into a total 1458 bins and was subjected for WebMGA analysis for COG percent categorization. ClustAGE took a set of nucleotide sequences of accessory genomic elements (AGEs) and clustered them to recognize the minimum set of accessory genomic elements in the population as well as determined the distribution of each accessory genomic element among the genomes. The outermost ring seen in Fig. (**5A**) was the bin category distributions of the genetic elements which was found to be dominated by putative solute binding proteins, and type IV secretory pathway, TrbL components. Other COG category percentage obtained through WebMGA analysis had been represented in the pie chart of Fig. (**5B**).

From the overall *in silico* study, it was observed that key clue on uniqueness of *B*. *anthracis* PFAB2 lies on its prophage region. And analyzing the result of PHASTER shown in Fig. (**6**), it was detected that all *B*. *cereus* isolates from international space station shared complete prophage region of *Bacillus* phage Btcs33, a characteristic *B*. *thuringiensis* specific phage in accessory genomes, which is unique in *B*. *cereus* clade. The size range of these prophage sequences varied from 33.6-40.1 kb in all the strains. Also, accessory genomes of both *B*. *anthracis* Ames Ancestor and Sterne consisted of a 37 kb complete prophage sequence of *Bacillus* phage vBBhaS. Other than this, the presence of complete prophage sequences of Bacillus phage PfEFR-5 and *Bacillus* phage phi4J1 had been also detected in different *B*. *anthracis* strains. Thus, prophage sequence pattern clearly differentiated between the closely related *B*. *anthracis* and *B*. *cereus* members. Most importantly, amongst the entire dataset of 15 strains of *B*. *cereus* and *B*. *anthracis*, complete prophage sequences are present in all reference strains, except two environmental variant of *B*. *anthracis*; PFAB2 and F34. Out of the entire dataset considered for the *in silico* study, *B*. *anthracis* PFAB2 and F34, these two strains exhibited no prophage region in accessory genome thus nullifying the occurrence of any possible lateral gene transfer events resulting into the absence of any virulence genes. The key genes required for *B*. *anthracis* to cause anthrax is reported to be acquired recently by horizontal gene transfer [37]. As both of the strains were isolated from highly extreme environments;

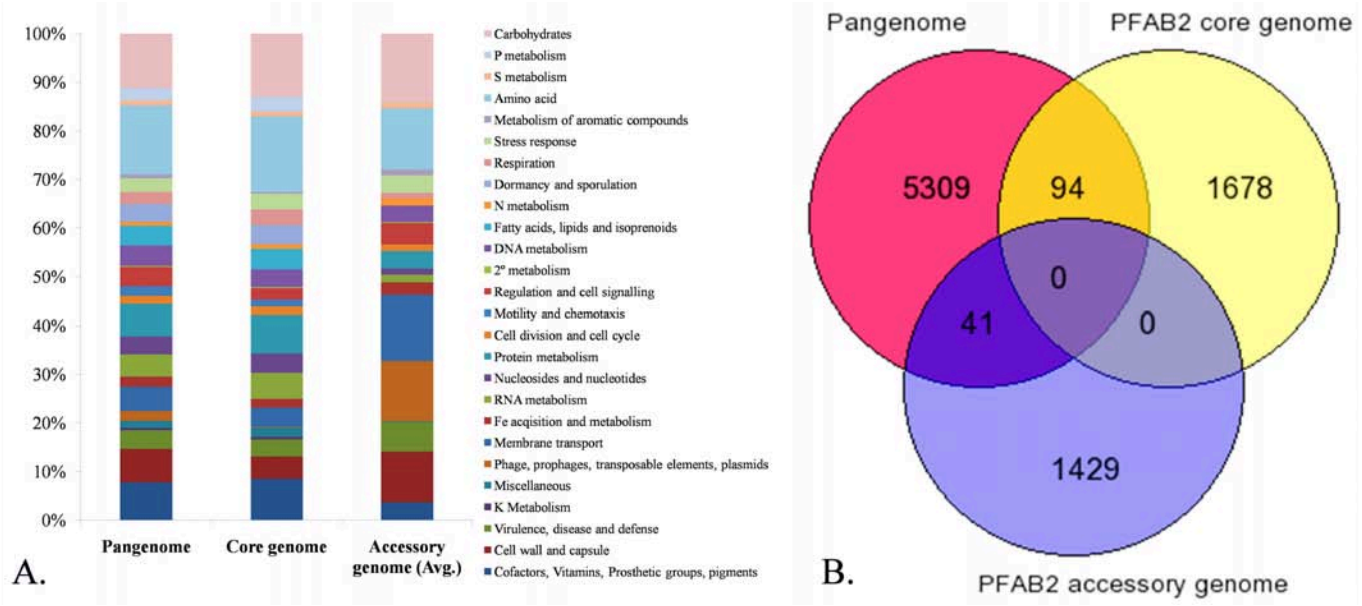


Fig. (4). A. Relative distribution of pan, core and accessory genome through Spine and Agent tool; where each category or subcategory is plotted as a percentage of the total number of genes in the core or accessory genomes and accessory genome percentages are averages of 15 reference strains; **B**. Venn diagram showing the relationship between pan, core and accessory genomes of *B*. *anthracis* PFAB2. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

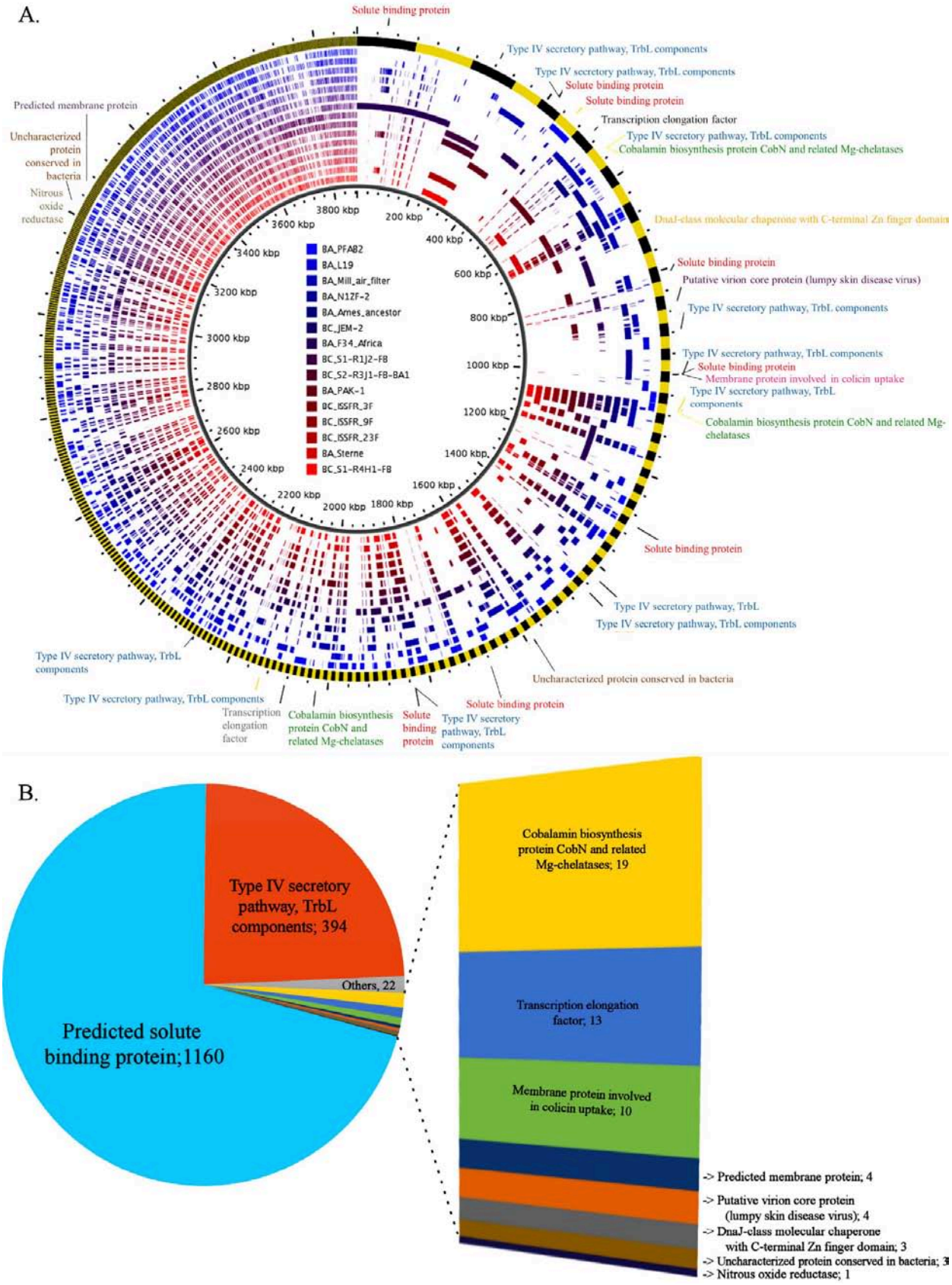


Fig. (5). **A**. Subelement classification of the 15 strains of *B. anthracis* and *B. cereus* including *B. anthracis* PFAB2, **B**. Pie chart showing the COG subcategorization pattern of the subelement clusters. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

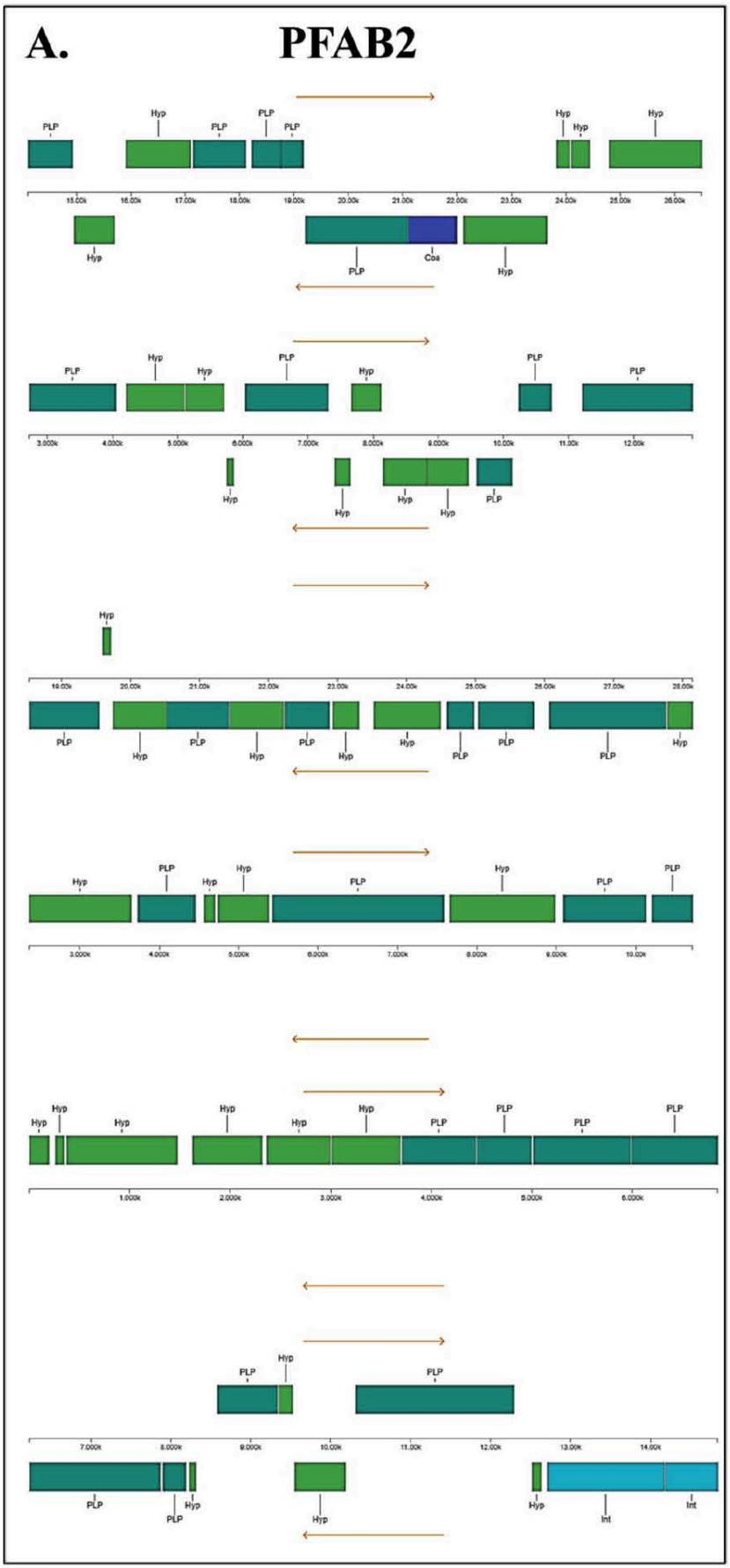
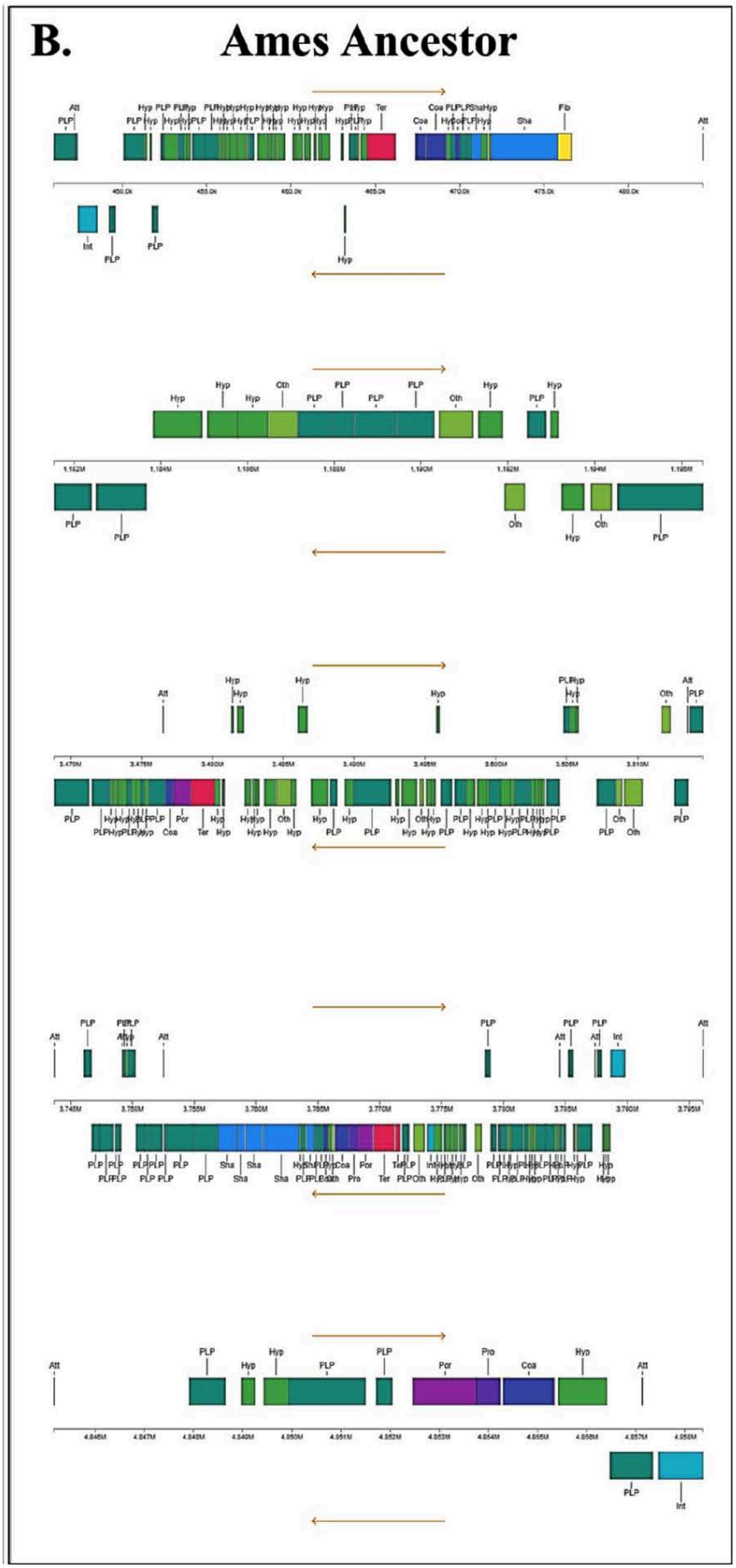


Fig. (6) contd….



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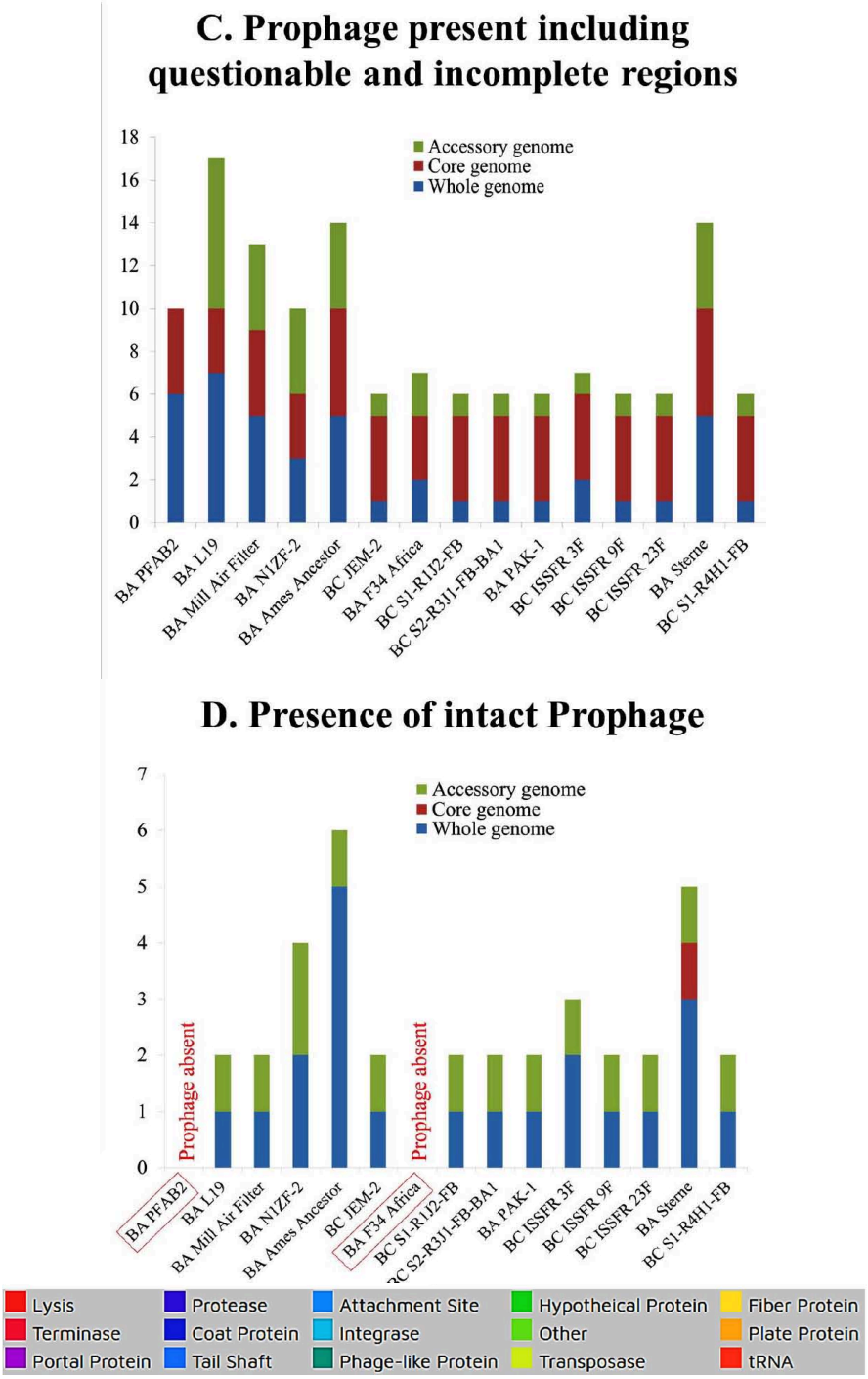

Fig. (6). Comparative analysis of prophage genes between *B. anthracis* PFAB2 (**A**) and reference *B. anthracis* Ames Ancestor (**B**), (**C**). Incomplete prophage sequences present in all reference strains, (**D**). PFAB2 and F34 exhibiting no prophage region in accessory genome. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

viz., extreme heat (PFAB2 from hot spring) and extreme salt concentration (F34 from salt lake), probably they never came across with any phage infection event. Thus near-clonal spread of anthrax-causing organism can be due to the expression of toxin and capsule genes by means of phage mediated transfer conferring an advantage to *B. anthracis*. This theory can be supported by a recently reported study by Papazisi *et al.* [38] where the acquisition of the plasmid-encoded pathogenicity island (pXO1) and capsule genes (pXO2) represented the crucial divergence of pathogenic *B. anthracis* from its close relatives.

3.6. Study of Growth Curve and Thermotolerance

Optimum temperature of *B. anthracis* is 37ºC for their growth and physiology. Thus, in order to understand the physiology of hot spring origin *B. anthracis* PFAB2, growth was observed at different temperatures in parallel with control Sterne. Sterne had exhibited slow growth pattern and

metabolic rate compared to PFAB2 in 37ºC indicating fast cell division potential of PFAB2. While increase in temperature visibly ceased the growth of Sterne, PFAB2 continued its growth till 50ºC and remained viable even in 55ºC confirming its thermotolerant nature (Fig. **7**). As most of the *B. anthracis* strains favored 37ºC for optimum growth, thermotolerant growth pattern of PFAB2 was probably supported by chromosomal genome only, as PFAB2 does not harbor plasmids. Origin in a hot spring with 66.2ºC temperature could be a source for addition, modification or activation of existing gene of PFAB2 to make them uniquely thermotolerant. Several heat shock proteins were also earlier reported from the whole genome of PFAB2 [13] that might be involved in controlling its thermotolerance *via* some unknown molecular switching mechanism.

3.7. Biofilm and Pellicle Formation Study

For survival and growth under adverse environmental conditions, bacteria are known to produce biofilm [39]. Thus, the extent of both biofilm and pellicle formation were investigated under static conditions at variable temperatures with oxygen deficiency. It was observed that Sterne was showing 25% reduction in biofilm formation as compared to PFAB2 at 37ºC. While under room temperature, ~25% decrease in biofilm formation was observed by PFAB2 compared to Sterne. Rate of biofilm formation negligibly changes for Sterne due to switching of temperature from RT to 37ºC; but increase in temperature significantly increased the rate of biofilm formation for PFAB2 (Fig. **8**). PFAB2 was also produced considerably more pellicle at 37ºC than RT; while Sterne showed less pellicle formation than PFAB2. Pellicles may be formed as a response to oxidative stress. Oxidative stress is also a common extreme factor in high temperature hot springs. Noticeable change in shape and size of *B. anthracis* pellicle cells were observed for both the strains under confocal microscope. While at normal growth condition, the cells were in clear dividing stage and smaller in size; a trend of chain formation was observed for pellicle cells which was also thinner in width. This type of characteristic chain formation by *B. anthracis* cells was earlier reported by Aggarwal *et al*. [30]. The positive effect of temperature on biofilm and pellicle formation can be inferred from the thermotolerant growth pattern of PFAB2.

3.8. Protein and Toxin Estimation in the Secretome

B. anthracis secreted toxins in culture media supernatant (CMS) are its primary virulence factors PA, LF, and EF [40, 41]. Out of all secreted proteins, PA with LF form lethal toxin, while PA with EF form edema toxins that show cytotoxic effect on macrophage cells [42]. CMS of any pXO1 plasmid harboring *B. anthracis* shows cytotoxicity on RAW macrophage cells. In MTT assay, quite high percent survival ratio (68.9%) was observed when macrophage cells were treated with PFAB2 CMS compared to the CMS of control strain Sterne (50.7%) (Fig. **9**). When the macrophage cells were treated with the same volume of CMS of different strains, percent survival of the cells depicts the pathogenicity of the strain and in case of PFAB2, ~70% survival ratio again confirms its minimal cytotoxic effect.

Further, the result was also justified by evaluating major toxicity factors PA, LF and EF *via* ELISA under ambient

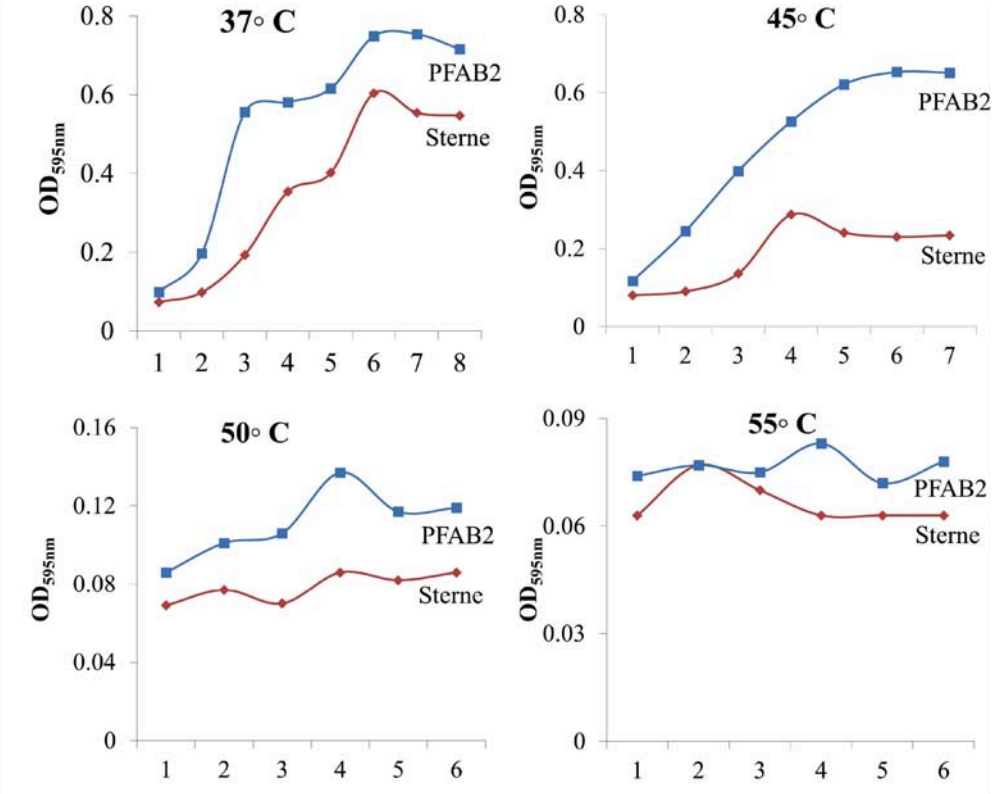

Fig. (7). Growth kinetics of *B. anthracis* PFAB2 in increasing temperature from 37ºC-55ºC in comparison with control *B. anthracis* Sterne. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

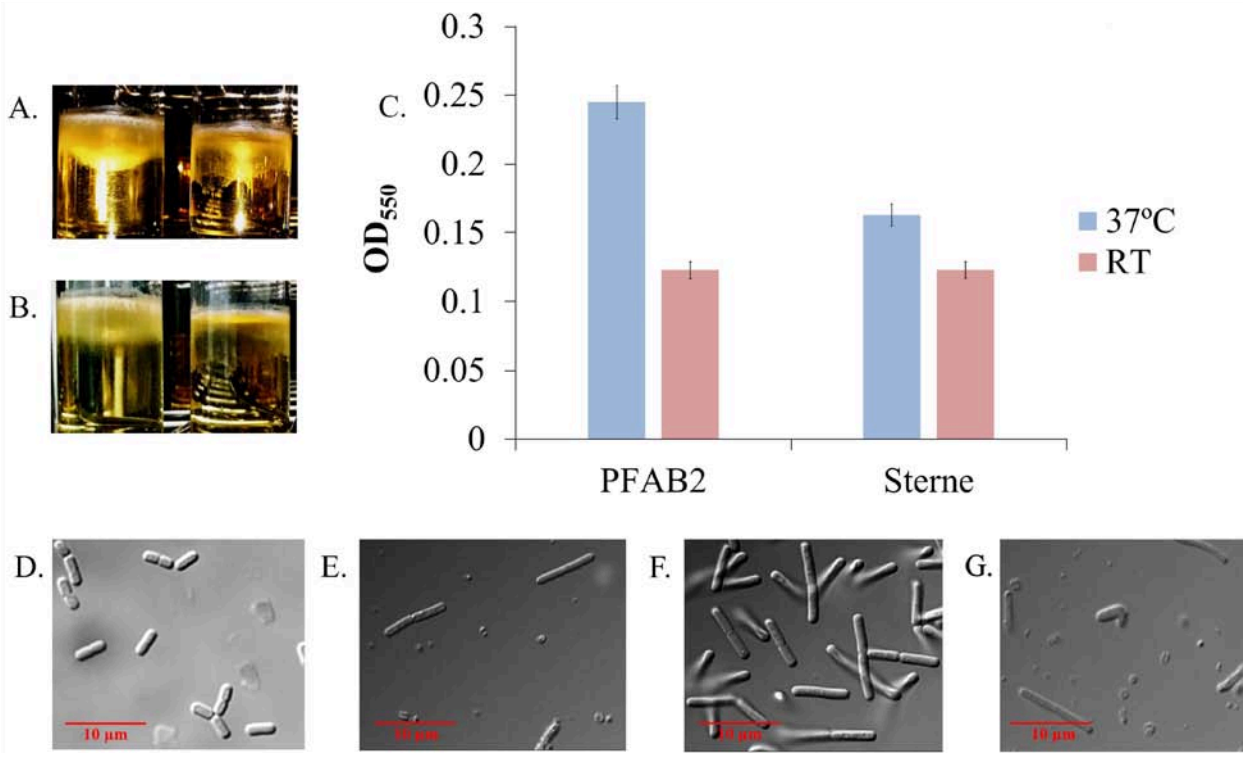

Fig. (8). Effect of temperature change on biofilm and pellicle formation when *B. anthracis* cells are allowed to grow under static conditions for 120 h. **A**. Pellicles of PFAB2 and Sterne respectively at 37ºC, **B**. Pellicles at RT, **C**. Graph showing the rate of biofilm formation under temperature change, **D**. Normally grown PFAB2 cells, **E**. PFAB2 pellicle cells, **F**. Normally grown Sterne cells, and **G**. Sterne Pellicle cells. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 1. Result of Protective Antigen (PA), Lethal Factor (LF) and Edema Factor (EF) toxin detection using ELISA.

Strain	PA	LF	EF	FlotP
PFAB2	-	-	-	+
Sterne	+	+	+	+

Where "-'' means absent and "+" present.

growth conditions. No production of PA, LF and EF toxins was detected by *B. anthracis* PFAB2 but the control strain Sterne has produced all the characteristic toxins (Table **1**).

3.9. *In Vitro* and *In Vivo* Pathogenesis Study

In order to investigate the virulence of *B. anthracis* PFAB2, association as well as internalization efficiency of PFAB2 was compared to that of the control *B. anthracis* Sterne within RAW macrophage cells by performing *in vitro* infection with log phase bacteria. Rate as well as capacity of internalization or invasion for PFAB2 was found to be almost half in comparison to the control Sterne. Additionally, association capacity of PFAB2 was also insignificant compared to association capacity of Sterne (Fig. **9A** and **9B**). Both the value predicts PFAB2's inability to become invasive and adhesive up to 4 h of macrophage infection.

Further with *in vivo* study, avirulence of *B. anthracis* PFAB2 was also confirmed in Swiss albino mice. These mice were infected with equal number of PFAB2 and Sterne bacilli (2×10^6) belonging to mid-log phase and survival of mice were analyzed for next 10 days. All the mice infected with Sterne were found to be dead within 5 days, whereas all the mice infected with PFAB2 were living and healthy until 10 days (Fig. **9D**). Thus, this study confirms the non-pathogenicity of PFAB2 (Fig. **9C** and **9D**).

3.10. Spore Formation and Germination Study

The primary and most crucial step in *B. anthracis* life cycle is formation and germination of its major infectious particle called spores. Earlier it was reported that steps of spore germination involved stable conversion from heat resistance to heat sensitivity [43, 44]. Different stages of spore formation by PFAB2 from vegetative cell to complete endospores were observed under transmission electron microscope. For measuring the germination potential of *B. anthracis* PFAB2 spores, CFU of the heat treated samples was calculated for every 5 min interval till 30 min. Spores of PFAB2 germinated at significantly higher

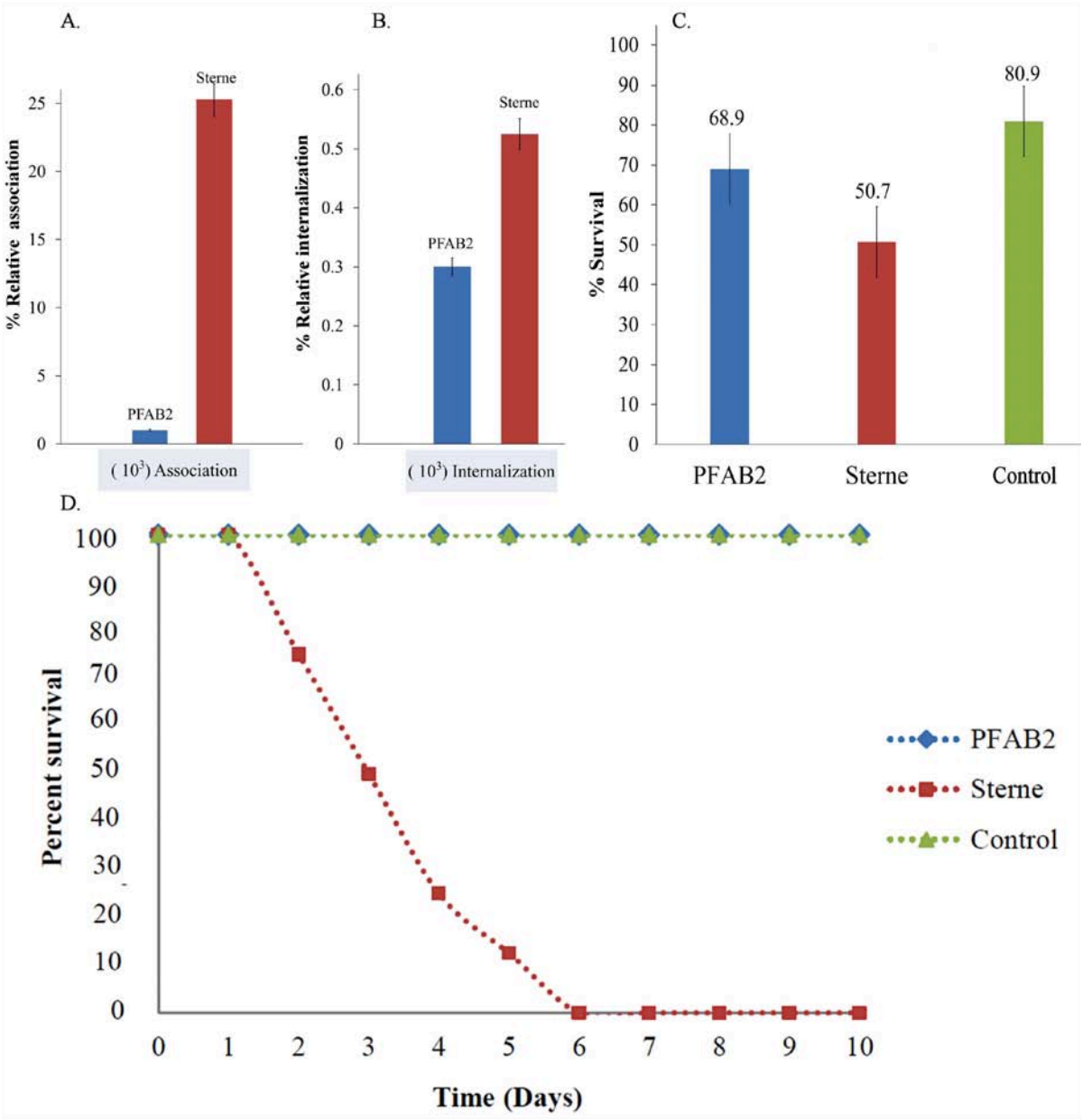

Fig. (9). RAW macrophage cell line study showing relative association (**A**) and internalization (**B**) of PFAB2 cells compared to control *B. anthracis* Sterne, (**C**). Percent survival of macrophages during PFAB2 and Sterne infection through MTT cytotoxicity assay compared to control, and (**D**). Kepler-Miller death plot showing the percent survival of the infected mice when treated with equal CFU of Sterne, PFAB2 and control blank media. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Summary of spore forming capacity of PFAB2 compared to Sterne.

Strain	Number of Spores (CFU/ml)	Genetic Composition	Occurrence
PFAB2	7×10^6	Genome+/pXO1-/pXO2-	Unique hot spring origin thermotolerant strain.
Sterne	3.2×10^6	Genome+/pXO1+/pXO2-	Well known laboratory strain.

speed compared to Sterne and was also almost double to that of Sterne (Fig. **10**). By CFU plating method, the same rate of spore forming capacity of PFAB2 was noticed. While Sterne produced 3.2×10^6 CFU/ml spores, PFAB2

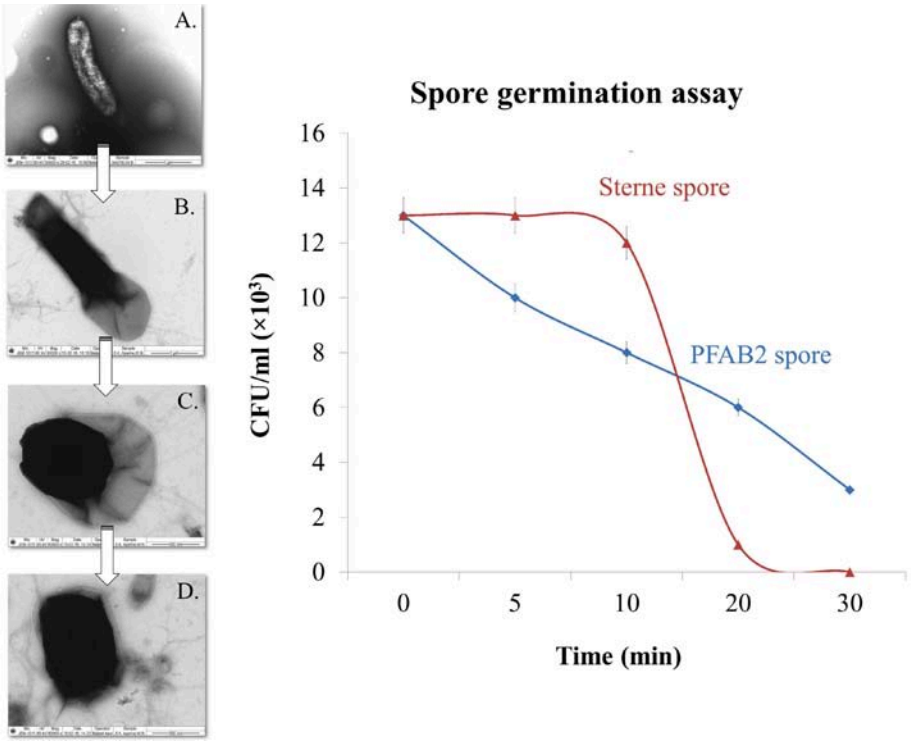

Fig. (10). Different steps of spore formation in *B. anthracis* PFAB2 as observed through transmission electron microscopy (in left) and rate of spore germination of PFAB2 compared to vegetative PAFB2 bacilli and control Sterne spores, when 5×10^3 cells are incubated in BHI media (in right). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

produced almost double the amount of spores (7×10^6 CFU/ml) (Table **2**).

Interestingly, the spore size of PFAB2 appeared to be 1.31 ± 0.08 µm in length; which was highly similar to an attenuated *B. anthracis* strain; Pasteur. Earlier reports suggested the spore size of Pasteur as 1.23 ± 0.11µm in length. *B. anthracis* strains that are shorter in length are generally attenuated *anthracis* strains and they sporulate much faster giving high yield compared to the virulent *B. anthracis* varieties that produce ~1.5 µm length spores [45, 46]. Surprisingly, being an avirulent variant of *B. anthracis*, PFAB2 had exhibited almost double the rate of spore formation and germination than Sterne. It may be probably due to the ability of PFAB2 to tolerate extreme temperature thus conferring better survival strategy. It can be also concluded that spore size is inversely proportional to the spore yield rate as well as germination for *B. anthracis*. Lower size probably helped bacterium to become more potent in becoming pathogenic but lack of plasmids again restricted the same.

CONCLUSION

PFAB2 is the first reported strain of *B. anthracis* isolated from a hot spring. This particular strain is unique in terms of its avirulence, thermotolerant growth pattern, high cell division rate and fast spore germination potential compared to all previously reported close variants of *B. anthracis*. In the present study, PFAB2 exhibited closeness with Ames Ancestor with 97.93% genomic identity as well as numerous physiological similarities except the pathogenesis. Both the *in silico* and *ex silico* outcomes signified PFAB2's future prospects in the investigations of hidden evolutionary as well as genome associated pathogenic aspects. One possible factor contributing to the unique avirulence of PFAB2 can be some possible plasmid curing events happened under stress condition of hot water spring. It was noticed that no complete prophage was present in accessory genomic region of PFAB2. This hinted no probable transduction events in past and nullified all the possibilities of lateral transfer of virulence-related genes. Complete absence of capsule biosynthesizing genes and plasmids in PFAB2 is a probable explanation for spread of non-pathogenic variant of *B. anthracis* from extreme environment to other natural surroundings due to possible phage infection events happened at a later stage of evolution. PFAB2-like strains can be the result of modification of *B. cereus* group from which pathogenic Ames Ancestor like *B. anthracis* evolved. Thus, occurrence of pathogenic *B. anthracis* likely to happen from a non-pathogenic *B. anthracis*. Strain like PFAB2 can be particularly of scientific interest as it may serve as a potential missing link between *B. cereus* and *B. anthracis*. It also favors the theory of earth's life evolution; where life has initially started in an extreme hot environment. Thus, this atypical variant of *B. anthracis*, strain PFAB2 may be used as a model *B. anthracis* system for better understanding on the evolution of anthrax virulence. Further studies are needed in order to achieve a conclusion on the evolution of pathogenic *B. anthracis*.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Animal experiments are approved by the Institutional Animal Ethics Committee of Jawaharlal Nehru University,

India (IAEC no. 45/2018) and conducted at the animal facility of the university under standard laboratory conditions.

HUMAN AND ANIMAL RIGHTS

No humans were used for study that are the basis of this resarch. The animal study is followed according to the CPCSEA guidelines for laboratory animal facility.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available in the [Genbank] at [https://www.ncbi.nlm.nih.gov/ assembly/GCF_001758315.1/], reference number [ASM175831v1], public release date [12-10-2016].

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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